Attachment 1

Neural Plasticity to Stress and Antidepressant Treatment

Ronald S. Duman, Jessica Malberg, and Johannes Thome

Adaptations at the cellular and molecular levels in response to stress and antidepressant treatment could represent a form of neural plasticity that contributes to the pathophysiology and treatment of depression. At the cellular level, atrophy and death of stress-vulnerable neurons in the hippocampus, as well as decreased neurogenesis of hippocampal neurons, has been reported in preclinical studies. Clinical studies also provide evidence for atrophy and cell death in the hippocampus, as well as the prefrontal cortex. It is possible that antidepressant treatment could oppose these adverse cellular effects, which may be regarded as a loss of neural plasticity, by blocking or reversing the atrophy of hippocampal neurons and by increasing cell survival and function. The molecular mechanisms underlying these effects are discussed, including the role of the cAMP signal transduction cascade and neurotrophic factors. Biol Psychiatry 1999;46: 1181–1191 © *1999 Society of Biological Psychiatry*

Key Words: Norepinephrine, serotonin, neurotrophic factors, neurogenesis, cyclic AMP, phosphodiesterase, MAP kinase

Introduction

An evolving hypothesis of the pathophysiology and treatment of depression involves adaptation or plasticity of neural systems. Depression could result from an inability to make the appropriate adaptive responses to stress or other aversive stimuli. This could be attributed to dysfunction of the normal mechanisms underlying neural plasticity. Antidepressant medications may act by correcting this dysfunction or by themselves directly inducing the appropriate adaptive responses. A role for plasticity in the actions of antidepressant treatment has been recognized for quite some time. This is based on the observations that the therapeutic action of antidepressants requires long-term administration even though these treatments block the reuptake or metabolism of norepinephrine (NE) and serotonin (5-HT) much more rapidly. This suggests that adaptations or plasticity to the acute actions of antidepressants are required.

A role for neural plasticity also indicates that the underlying mechanisms for the therapeutic action of antidepressants, as well as the etiology of depression, are much more complex than simply changing synaptic levels of monoamines. Recent studies have begun to characterize adaptations of neuronal morphology and survival at the cellular level, and the intracellular signal transduction cascades at the molecular level, that underlie the response to antidepressant treatments. Our hypothesis proposes that these adaptations oppose the actions of stress and environmental factors, as well as genetic factors, that lead to depression. In this review, we will describe some of actions of stress and antidepressant treatments on hippocampal neurons and the intracellular mechanisms that are thought to underlie these effects.

Evidence for Neuronal Atrophy and Loss of Plasticity in Response to Stress

Stress is known to influence a wide range of neuronal systems that in the acute phase result in beneficial endocrine and behavioral responses; however, repeated or severe stress or increased vulnerability due to genetic factors can lead to adverse effects on neuronal function. The hippocampus is one brain structure that has been extensively studied with regard to the actions of stress, depression, and antidepressant actions. Dysfunction of the hippocampus could result in some of the vegetative and endocrine abnormalities, as well as cognitive and memory deficits, observed in depressed patients. Hippocampal neurons are reported to be damaged by exposure to stress or activation of the hypothalamic-pituitary-adrenal (HPA) axis and elevation of glucocorticoids.

Stress Results in Atrophy and Death of CA3 Pyramidal Neurons in the Hippocampus

CA3 pyramidal neurons in the hippocampus have been demonstrated to be extremely vulnerable to stress and

From the Laboratory of Molecular Psychiatry, Departments of Psychiatry and Pharmacology, Yale University School of Medicine, Connecticut Mental Health Center, New Haven, Connecticut.

Address reprint requests to Dr. Ronald S. Duman, 34 Park Street, New Haven, CT 06508.

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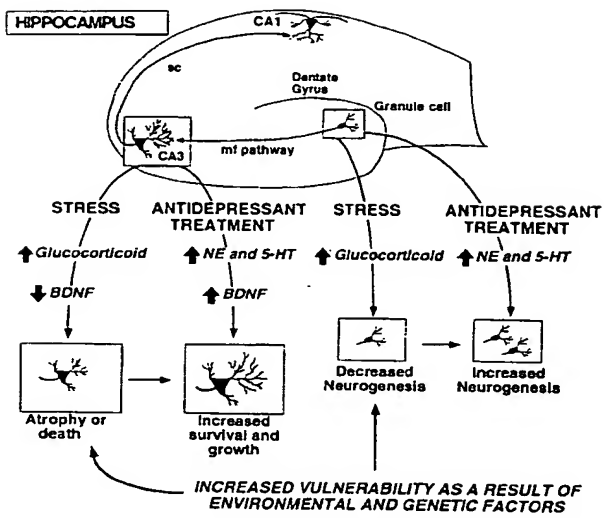

Diagrammatic representation of the actions of stress and antidepressant treatment on hippocampal neurons: A molecular and cellular hypothesis of depression. Stress or glucocorticoid treatments are reported to cause atrophy and, in severe cases, death of CA3 pyramidal neurons and to decrease neurogenesis of dentate gyrus granule cells in the hippocampus. These effects of stress and glucocorticoid treatments on CA3 neuronal atrophy and death are thought to be mediated by reduced glucose transport, increased glutamate and Ca^{2+} excitotoxicity, and decreased expression of BDNF. Glucocorticoids are known to contribute to the stress-induced down-regulation of neurogenesis. It is possible that these effects contribute to the atrophy of the hippocampus observed in depression. Individual vulnerability to stress and depression could result from environmental or genetic factors that influence neuronal atrophy and survival. This model also proposes that antidepressant treatments oppose these actions of stress and either block or reverse hippocampal atrophy and cell death. The mechanisms underlying such actions of antidepressants have not been well defined but may involve up-regulation of BDNF via increased NE and 5-HT signal transduction.

adrenal glucocorticoids (Figure 1) (for core references and complete reviews see Sapolsky 1996a; McEwen 1999). The adverse effects of chronic exposure to stress on these neurons can be subdivided into three areas. First, atrophy of CA3 neurons has been reported in both rodents and nonhuman primates after exposure to repeated restraint stress. This effect is also observed in response to administration of glucocorticoids at a dose that approximates levels that are induced by stress. Atrophy is demonstrated by a decrease in the number and length of branch points of the apical dendrites of CA3 neurons. Second, death of CA3 neurons has been reported to occur in response to severe and long-term stress or glucocorticoid treatment. Finally, neuroendangerment of CA3 neurons by exposure to stress has been demonstrated. In this case, acute exposure to stress or glucocorticoids exacerbates the damage of CA3 neurons caused by other neuronal insults, such as hypoglycemia, hypoxia, or excitotoxins.

Stress Decreases Neurogenesis of Dentate Gyrus Granule Neurons in the Hippocampus of Adult Animals

Although dentate gyrus granule neurons in the hippocampus appear to be relatively resistant to atrophy and death, stress is reported to decrease the birth or neurogenesis of these cells in adult animals (Figure 1). Although the capacity for new cell birth is not observed in most regions of the mature nervous system, the dentate gyrus is one of the few areas where adult neurogenesis has been demonstrated. Neurogenesis is studied by labeling the DNA of dividing cells with radiolabeled thymidine or a thymidine analogue, bromodeoxyuridine (BrdU). Cells can then be visualized by autoradiography or immunohistochemistry, respectively. Adult neurogenesis of granule cells has been reported in rodents and nonhuman primates and more recently in humans (Gould et al 1997, 1998; Ericksson et al 1998; see Greenough et al 1999). Progenitor cells located in the subgranular zone proliferate and migrate into the granule cell layer and hilus. Among the factors demonstrated to influence the rate of neurogenesis are glucocorticoids. Normal rates of neurogenesis, as well as death of granule cells, are dependent on physiological concentrations of glucocorticoids; however, acute stress or exposure to high levels of glucocorticoids decreases neurogenesis of granule cells (Gould et al 1997, 1998).

Although the exact function of neurogenesis has not been determined, increases in the rate of granule cell birth and survival of these neurons are associated with an enriched environment and training in models of learning and memory. Gage and colleagues have reported in two studies that exposure of adult mice to an enriched environment (increased contact with inanimate objects and enhanced social interactions with other mice) increases granule cell neurogenesis (Kempermann et al 1997; van Praag et al 1999). In addition, one study has demonstrated that training of animals in an associative learning task increases the survival of newly formed cells (Gould et al 1999). Although a similar study did not observe an increase of neurogenesis in response to a learning task, this could be related to the experimental design (see Greenough et al 1999). These studies raise the possibility that increased birth and survival of granule cells could contribute to learning, as well as other behavioral and endocrine functions under the control of the hippocampus. Moreover, down-regulation of neurogenesis in response to stress could contribute to deficits in the functional capacity of the hippocampus.

Clinical studies have demonstrated that the size and function of the hippocampus are reduced in patients with depression. Brain imaging studies have reported that the volume of the hippocampus is reduced in patients with depression or PTSD (Sheline et al 1996; Bremner et al 1999; also see Sapolsky 1996b for review). Decreased hippocampal volume is also reported in patients with Cushing's disease, indicating that elevated glucocorticoid levels underlies the reduced volume. This effect has also been shown to be reversible in patients with Cushing's disease, upon normalization of glucocorticoid levels. Studies are currently underway to determine if the volume reduction observed in depression is reversed in patients in remission. Elevation of cortisol levels in elderly patients also correlates with reduced hippocampal volume and is associated with memory deficits (Lupien et al 1998). Studies of hippocampal feedback inhibition of the HPA axis have also demonstrated a functional deficit in patients with depression (Young et al 1991). Glucocorticoid-induced loss of negative feedback could represent a downward cycle of reduced hippocampal function and damage that leads to a further loss of feedback inhibition and elevation of adrenal-glucocorticoids.

Although these clinical studies are consistent with basic research studies, more direct analysis of neuronal atrophy and survival in patients with depression is needed. Postmortem studies of the number of CA3 pyramidal cells and dentate gyrus granule cells in the hippocampus will be necessary to begin to characterize the cellular mechanisms that underlie the reduction in volume and function of the hippocampus.

Studies of the prefrontal cortex demonstrate that atrophy and death of neurons also occurs in other brain regions thought to be involved in depression and mood disorders. Brain imaging studies have demonstrated a reduction in blood flow and volume of the prefrontal cortex (Drevets et al 1997). Moreover, two recent studies report that the number of cells in prefrontal cortex are decreased in patients with depression. The first of these studies reports a reduction in the number of glia, but not neurons, in the subgenual prefrontal cortex of patients with major depressive disorder or bipolar disorder (Ongur et al 1998). A second study has reported a decrease in neuronal size and the number of neurons and glia in the prefrontal and rostral orbitofrontal cortex (Rajkowska et al 1999). These findings suggest that atrophy and survival of neurons may also contribute to certain symptoms of depression, such as depressed mood and working memory, that can be attributed to prefrontal cortex. Additional preclinical studies at the molecular and cellular levels are needed to understand how antidepressant agents may influence neuronal systems in the prefrontal cortex.

Molecular Mechanisms Underlying the Actions of Stress

The influence of stress and glucocorticoids on neuronal atrophy and survival could involve multiple complex and overlapping intracellular pathways. Studies of these pathways have identified three major effects, including uptake and metabolism of glucose, increased glutamate and Ca^{2+} excitoxicity, and down-regulation of neurotrophic factors, that could contribute to the actions of stress (for review see Sapolsky et al 1996a). A brief review of these mechanisms is provided here. In addition, it is possible that there is dysregulation of the pathways that control programmed cell death, or apoptosis, in depression. This could also account for loss of neurons and glia observed in prefrontal cortex of patients with depression. There are several reports demonstrating that lithium reduces apoptosis of cerebellar granule neurons in culture (Nonaka et al 1998; Chen and Chuang 1999). This is a potentially interesting area of research for studies of the pathophysiology and treatment of depression that warrants future consideration.

Influence of Stress on Glucose Uptake and Glutamate-Induced Excitoxicity

Glucocorticoids are known to influence glucose uptake and cellular energy in peripheral tissues, and similar effects could contribute to the influence of stress on neuronal atrophy and survival. Glucocorticoids also are reported to decrease the uptake of glucose in fat cells, and a similar effect is observed in primary neuronal cultures (Horner et al 1990). Although the exact mechanisms have not been determined in the brain, studies in fat cells demonstrate that glucocorticoid exposure decreases the expression of the glucose transporter and induces a translocation of the transporter from the cell membrane to an intracellular compartment (see Sapolsky 1996a). Both of these effects would contribute to a reduction in glucose uptake in cells. This could result in decreased cellular metabolism that could eventually cause neurotoxicity or produce a heightened state of neuroendangerment to other types of insult (e.g., excitotoxins, hypoxia, and hypoglycemia).

Another mechanism that is thought to be involved in the actions of stress and glucocorticoids is increased glutamate and calcium excitotoxicity. Stress or glucocorticoid

administration is reported to increase levels of glutamate in extracellular dialysate in the hippocampus (see Sapolsky 1996a). Glutamate, acting via NMDA and non-NMDA ionotropic receptors, increases intracellular levels of Ca^{2+}, and sustained activation of glutamate-induced Ca^{2+} is known to underlie the excitoxic effects of repeated seizures and ischemia. Based on these observations, it has been suggested that enhanced glutamate release could also contribute to glucocorticoid-induced neuroendangerment. Several questions remain to be answered regarding the role of glutamate and calcium in the actions of stress, however. For example, the expression of immediate early genes, such as Fos, that are typically induced in the hippocampus by stimuli that release glutamate (such as seizure or ischemia) are not induced by stress. Second, there is some evidence that elevation of glutamate could occur in response to placement of the dialysis probe (see Lowy et al 1995). In spite of these questions, elevation of glutamate and intracellular Ca^{2+} remains a viable potential mechanism that could contribute to stress- and glucocorticoid-induced endangerment and toxity of hippocampal neurons.

Expression of Brain Derived Neurotrophic Factor (BDNF) Is Down-Regulated by Stress

BDNF belongs to the neurotrophic factor family, which also includes nerve growth factor, neurotrophin-3, and neurotrophin-4/5 (Thoenen 1995). Neurotrophic factors were originally characterized based on their ability to influence the differentiation and development of specific populations of neurons in the nervous system. More recent studies demonstrate that these factors also influence the survival and function of neurons in the mature, adult brain. The expression of BDNF is induced in response to neuronal activity, for example, and has been shown to play a critical role in cellular models of learning and memory (i.e., long-term potentiation or LTP).

Evidence for the involvement of BDNF, and possibly dysfunction of other neurotrophic factor systems, in responses to stress have also been reported. Smith and colleagues have demonstrated that immobilization stress decreases the expression of BDNF in hippocampus (1995). In this report, acute or repeated exposure to immobilization stress decreased levels of BDNF mRNA in the major subfields of hippocampus (CA1 and CA3 pyramidal and dentate gyrus granule cell layers). We have replicated this effect and also found that other types of stress decrease the expression of BDNF (Nibuya et al 1999). Decreased expression of BDNF could contribute to the adverse effects of stress on hippocampal neurons (see below).

The mechanisms underlying the influence of stress on BDNF expression have not been fully characterized. Administration of corticosterone produces a small decrease in levels of BDNF mRNA in the dentate gyrus, but not in CA1 or CA3 pyramidal cell layers (Smith et al 1995). Removal of the adrenal glands does not block the down-regulation of BDNF in dentate gyrus, although the effects in CA1 and CA3 are attenuated. These results suggest that the down-regulation of BDNF can be explained in part by elevation of adrenal-glucocorticoids but suggest that other factors also contribute to this effect of stress. Another possibility is that monoamine systems, such as NE and 5-HT that are activated by stress, could influence the expression of BDNF. We have found that pretreatment with a $5\text{-}HT_{2A}$ antagonist (i.e., ketanserin or MDL 100, 907) reduces by approximately half the down-regulation of BDNF (Vaidya et al 1997, 1999). Although the exact mechanism has not been determined, stress-induced down-regulation of BDNF in the hippocampus by $5\text{-}HT_{2A}$ receptors could occur via activation of GABAergic interneurons that inhibit hippocampal neuronal activity (see Vaidya et al 1997 and 1999 for further discussion). Because some antidepressant drugs exhibit antagonist properties for $5\text{-}HT_{2A}$ receptors, it is interesting to speculate that blockade of BDNF down-regulation could contribute to the action of these drugs. Additional studies will be required to determine if the glucocorticoid and $5\text{-}HT_{2A}$ receptor mechanism mediate the entire stress response or if other factors also contribute to this effect.

Neuronal Plasticity in Response to Antidepressant Treatment

These reports of atrophy and cell death in stress and depression raise the possibility that the action of antidepressants may involve reversal or blockade of these effects or direct regulation of synaptic architecture, dendritic morphology, and survival of neurons. In support of this hypothesis, several studies have reported that antidepressant treatments exert positive actions on these cellular processes. These studies have focused on the hippocampus, and future work will be required to determine the influence of antidepressants on cell survival in prefrontal cortex and other brain regions.

Influence of Antidepressant Treatment on Atrophy of Hippocampal Neurons

Tianeptine has the unusual property of

enhancing reuptake of 5-HT. Administration of either drug alone did not influence the morphology of the apical dendrites of these neurons. Further studies are required to determine if other classes of antidepressant drugs or different treatment regimens for the 5-HT selective reuptake inhibitors can block the atrophy of hippocampal neurons in response to stress.

Influence of Antidepressant Treatment on Hippocampal Neurogenesis

Another mechanism by which antidepressant treatment could oppose the actions of stress is via up-regulation of the neurogenesis of dentate gyrus granule neurons. Preliminary studies from our laboratory indicate that chronic, but not acute, antidepressant treatment increases neurogenesis of hippocampal granule cells (Duman and Malberg 1998). An increase in the number of BrdU-labeled neurons is observed in response to chronic administration of several different classes of antidepressants, including 5-HT and NE selective reuptake inhibitors, a monoamine oxidase inhibitor, and electroconvulsive seizures (ECS). Increased BrdU labeling in response to fluoxetine has also been observed by another group (Jacobs and Gould, Princeton University, unpublished observation). In a preliminary report, these investigators have also demonstrated that administration of a 5-HT1A agonist increases neurogenesis, suggesting that this receptor subtype could mediate the action of 5-HT; (Jacobs et al 1998). Although additional studies are required to determine if up-regulation of BrdU labeling is a result of increased cell birth or survival and to determine if the labeled cells are neurons or glia, elevated neurogenesis provides another mechanism by which antidepressant treatment could oppose the actions of stress.

Influence of Antidepressant Treatment on Sprouting of Hippocampal Neurons

Granule neurons in the hippocampus are also reported to undergo sprouting in response to excitotoxin treatment and kindling paradigms, and we have found that chronic ECS administration also induces sprouting of granule cells (Vaidya et al 1999). This effect is dependent on repeated ECS treatment and is long lasting (e.g., observed up to at least 6 months after the last ECS treatment). Excitotoxin- and kindling-induced sprouting are thought to be, at least in part, adaptations in response to death of target neurons (see Vaidya et al 1999). In contrast, there is no evidence of cell loss or dying neurons in response to chronic ECS. Alternatively, we have reported that ECS-induced sprouting is significantly attenuated in BDNF heterozygous knock-out mice, which express half of the normal level of BDNF. In this study, infusion of BDNF alone was not sufficient to induce sprouting, suggesting that BDNF is necessary but not sufficient to produce sprouting. Chronic administration of antidepressant drugs does not influence sprouting of granule cells, suggesting that this effect may be specific to ECS. Further studies are required to determine the functional significance of increased sprouting in the actions of ECS.

Intracellular Signaling Mechanisms

One signal transduction cascade implicated in the action of antidepressant treatment is the cAMP pathway. Although early studies demonstrate that β-adrenergic receptor (βAR) coupling to this second messenger system is decreased, more recent studies show that the postreceptor, intracellular components of the cAMP cascade are up-regulated by antidepressant treatment.

Adaptations of the Intracellular Components of the cAMP Pathway

(Figure 2). First, one study reported that coupling of the stimulatory G protein, Gs, to adenylyl cyclase is increased by chronic antidepressant treatment (Ozawa et al 1994). Second, levels of cAMP-dependent protein kinase (PKA) in particulate fractions of limbic brain are reported to be up-regulated (Nestler et al 1989; Perez et al 1989). One study has also reported that levels of PKA in the nuclear fractions of cerebral cortex are increased, suggesting that gene expression is regulated by antidepressant treatment (Nestler et al 1989). This is supported by the finding that expression of the cAMP response element binding protein (CREB), a transcription factor that mediates many of the actions of the cAMP system on gene expression, is also up-regulated by chronic, but not acute, antidepressant treatment (Nibuya et al 1996).

The mechanisms underlying increased expression of CREB mRNA and protein have not been elucidated in vivo, but cell culture studies demonstrate that this could occur via activation of the cAMP system (Widnell et al 1994). This implies that there is a positive feed-forward mechanism that regulates the expression and function of CREB. It is also possible that altered expression or function of CREB could contribute to the pathophysiology of depression. A recent postmortem study has demonstrated that the expression of CREB is decreased in the temporal cortex of patients with depression and that antidepressant treatment reverses this effect (Dowlatshahi et al 1998). It is also possible that CREB is reduced in response to the depressive state, however. Additional studies are required to confirm this observation in a larger

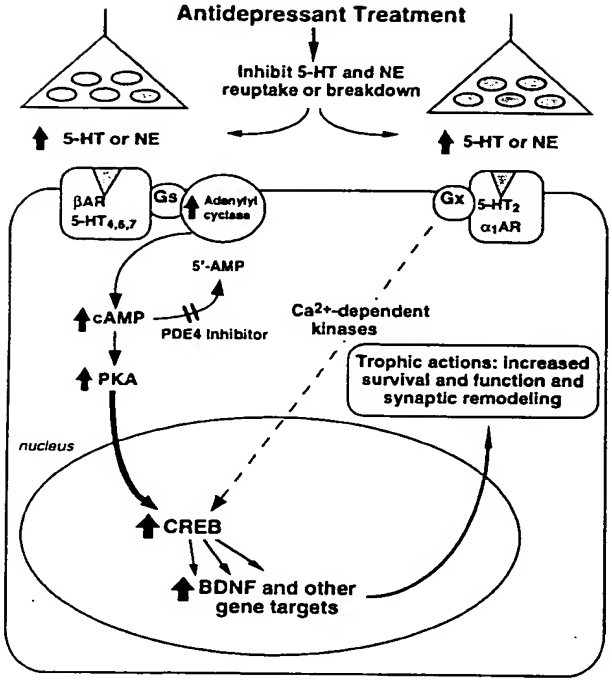

Figure 2. A model depicting the actions of antidepressant treatment on the cAMP signal transduction cascade. Chronic administration of several different classes of antidepressant is reported to up-regulate the cAMP system at several levels, including increased coupling of Gs and adenylyl cyclase, increased levels of cAMP-dependent protein kinase (PKA), and increased expression of cAMP response element binding protein (CREB). These findings suggest that the cAMP system and CREB may be a common postreceptor target of antidepressant treatment. In addition to activation of CREB by the cAMP system and receptors coupled to this pathway (e.g., βAR and $5\text{-HT}_{4,6,7}$), CREB can also be activated by Ca^{2+}-dependent protein kinases and receptors linked to these pathways (e.g., 5-HT_2 and β_1AR). These findings suggest that up-regulation of the cAMP system may contribute to the action of antidepressant treatment. This possibility is supported by studies demonstrating that inhibitors of cAMP phosphodiesterase (PDE4), the enzymes responsible for breakdown of cAMP, have antidepressant efficacy in behavioral models and clinical trials. Up-regulation of CREB also suggests that antidepressant treatment influences the expression of specific target genes, one of which is brain derived neurotrophic factor (BDNF).

sample size, to determine if a similar effect is observed in other brain regions, such as the hippocampus, and to determine if decreased CREB is a state or trait effect.

Is CREB a Common Postreceptor Target of Antidepressant-Induced Neural Plasticity?

Up-regulation of CREB occurs in response to chronic administration of different classes of antidepressants, including both NE and 5-HT selective reuptake inhibitors, supporting the possibility that CREB could be a common postreceptor target of antidepressant treatment (Nibuya et al 1996). CREB is a particularly interesting and viable common target because it can be activated by several different signal transduction pathways. The transcriptional activity of CREB is increased when it is phosphorylated at Ser133. In addition to phosphorylation by PKA, which could occur via NE (βAR) or 5-HT ($5\text{-HT}_{4,6,7}$) receptors, CREB can be phosphorylated and activated by Ca^{2+}-dependent protein kinases, including both Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C (Figure 2). These kinases could be regulated by NE and 5-HT receptors, such as the α_1-adrenergic or 5-HT_2 receptors coupled to the phosphatidylinositol system or by other receptors or ion channels that influence Ca^{2+} signaling. In addition, CREB can be phosphorylated by another kinase, referred to as rsk or CREB kinase, that is activated by the MAP kinase pathway (see below).

Although the studies cited support a role for the cAMP system and CREB in the action of antidepressant treatment, there are several points that must be addressed. First, it has been difficult, due to technical problems, to characterize the phosphorylation state of CREB in response to chronic antidepressant treatment. This is a critical point because it is possible that the expression of CREB is increased but that the phosphorylation state is reduced or unaltered. Studies are currently being conducted to address this point using CRE-lacZ transgenic mice that express a reporter gene under the control of a promoter-containing tandem CRE elements (Thome et al 1998). Preliminary studies demonstrate that chronic antidepressant treatment increases reporter gene expression in the CRE-lacZ mice, indicating that the phosphorylation and function of CREB are increased. A related question is how does an increase in the expression of CREB influence neuronal function and behavior. We are addressing this point by creating inducible and region-specific transgenic mice where the transgene is CREB or a dominant negative mutant of CREB (Chen et al 1998). This will allow us to regulate the expression of CREB or its negative mutant inhibitor in specific brain regions in adult animals and then test the functional consequences of altered CREB. These approaches will provide additional evidence to test the role of CREB in the actions of antidepressant treatment.

Activation of the cAMP Cascade as a Target of Antidepressant Treatment

The results of these studies indicate that activation of cAMP signaling and CREB could produce an antidepressant response. Support for this hypothesis has been provided by clinical studies with inhibitors of cAMP

phosphodiesterase (PDE4), the enzyme responsible for the breakdown of cAMP (Figure 2) (see Duman 1998 for review of basic and clinical studies). Administration of rolipram, a relatively selective cAMP PDE inhibitor, has been reported to have antidepressant efficacy in clinical trials, although this compound has not been developed because of its side effects. There are multiple isoforms of PDE4 that are inhibited by rolipram (see Conti and Jin 1999), however, and it is possible that selective inhibition of one of these could result in antidepressant actions without the side effects. We and others have provided evidence supporting a role of PDE4A and PDE4B (Ye et al 1997; Suda et al 1998; Takahashi et al 1999), although further studies are required to test the validity of these isozymes as targets for novel antidepressant medications. Another possibility for activating the cAMP cascade is via agonists for receptors directly coupled to this pathway, such as 5-$HT_{4,6,7}$ receptors. Stimulatory receptors, as well as intracellular sites, may represent novel targets for antidepressant drugs.

Adaptation of βAR-Stimulated cAMP Production

Although these more recent studies demonstrate up-regulation of the cAMP cascade, one of the first reported adaptations to antidepressants was down-regulation of βAR and the ability of these receptors to stimulate cAMP formation. This resulted in the βAR subsensitivity hypothesis, which stated that depression was a consequence of excess βAR and that antidepressants alleviate depression by reducing the number of receptors. In addition to the reports of an up-regulated cAMP cascade cited above, further evidence against this hypothesis has accumulated (see Duman et al 1997 for review). First, using more specific ligands, receptor binding studies have demonstrated that the down-regulation of βAR occurs much more rapidly (i.e., 1–3 days) than the therapeutic action of antidepressant treatment. Second, based on the subsensitivity hypothesis, antagonists of the βAR would be predicted to have antidepressant efficacy, but this has not been reported. In fact, there is evidence that βAR-antagonist treatment may actually produce depression in some patients. In contrast, there is evidence that increased expression and function of βAR may result in an antidepressant response. Administration of thyroid hormone, a treatment reported to increase the expression of βAR, is reported to have antidepressant efficacy.

Up-Regulated cAMP Cascade and Decreased βAR Coupling: Paradoxal Effects or Related Adaptative Responses?

The discovery that the cAMP cascade is up-regulated but that βAR-stimulated production of this second messenger is decreased appears to be contradictory; however, one problem with the βAR-stimulated cAMP assays is that they typically measure levels of receptor activation of the second messenger in an in vitro brain slice or homogenate. In these in vitro systems, the ongoing action of the antidepressant drug on levels of NE cannot be taken into account. In fact, endogenous NE is washed out of the preparations, and the influence of an exogenous agonist is determined. This is a critical point because it is possible that in vivo, even though levels of βAR are decreased by chronic antidepressant treatment, there are sufficient levels of the receptor remaining to produce an elevated intracellular response to the increased levels of NE (Figure 3). This could be the case when compared to the control condition, where there is a normal complement of receptor but where levels of NE are lower than under the antidepressant condition. Assuming this is the case, the responsiveness of the βAR-coupled cAMP system may be reduced relative to that observed in the presence of a maximum concentration of NE as reported in vitro, but in vivo the response would be higher than that observed in the absence of antidepressant treatment. Although there are no in vivo brain studies that address this hypothesis,

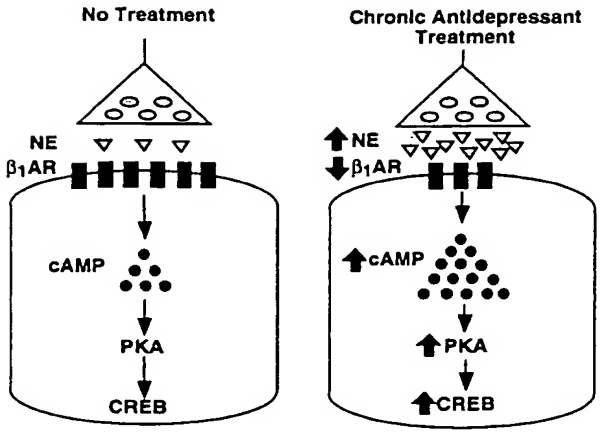

Figure 3. A model explaining the paradoxical up-regulation of the cAMP system and down-regulation of βAR binding sites in response to antidepressant treatment. In the absence of treatment, levels of NE and βAR-stimulated cAMP are relatively low. Chronic antidepressant treatment results in increased synaptic levels of NE and activation of βAR. This leads to down-regulation of βAR, although the receptors are not completely eliminated from the membrane. The model proposes that in the presence of elevated NE, there is sustained activation of the remaining receptors and increased levels cAMP relative to that observed in the absence of treatment. This is a hypothetical model that requires further testing, but it is consistent with results of studies demonstrating that the cAMP cascade is increased even though levels of βAR are decreased by antidepressant treatment.

reports on the influence of antidepressants on heart function are consistent with this idea. Basal heart rate is reported to be increased by chronic antidepressant treatment, even though the response to exogenous isoproterenol is decreased (Rosenstein and Nelson 1991; Roose et al 1998).

The notion that the cellular consequences of receptor activation cannot be predicted based on levels of receptor number is an important concept that could apply to many receptor systems. It is critical to remember that the cellular responses to receptor activation are also dependent on the in vivo levels of the neurotransmitter acting at the receptor, as well as the intracellular pathways that mediate the action of the receptor. These factors could apply to the interpretation of receptor binding studies in preclinical studies, as well as for human brain imaging studies of receptors.

Role of BDNF in the Action of Antidepressant Treatment

Up-regulation of the cAMP system and CREB suggests that antidepressant treatments regulate the expression of specific target genes. One gene target of interest is BDNF. Recent studies have provided evidence that up-regulation of BDNF may be involved in the actions of antidepressant treatment and that decreased expression of this neurotrophic factor could contribute to the negative influence of stress on certain neuronal systems.

Neurotrophic Factor Signal Transduction Cascade

The intracellular signal transduction pathways that mediate the actions of neurotrophic factors are fundamentally different from those for G protein coupled receptor-second messengers (Figure 4) (see Russell et al 1995). Neurotrophic factors act on receptors, referred to as Trks, that contain an extracellular binding domain and an intracellular tyrosinse kinase domain. There are at least three different Trk receptors that display some selectivity for the neurotrophic factors: TrkA for NGF, TrkB for BDNF, and TrkC for NT-3/NT-4/5. Binding of two molecules of neurotrophic factor results in receptor dimerization and activation of the intracellular tyrosine kinase domain of the receptor, resulting in phosphorylation of the receptor itself as well as other cellular effector proteins. Depending on the cell type, different effector pathways may be activated. These include phosphatidylinositol-3 kinase, phospholipase C-γ, and the MAP kinase signal transduction pathway. Activation of the MAP kinase pathway involves regulation of several intermediate steps. This includes tyrosine phosphorylation of adaptor proteins, Shc and Gab, and in conjunction with another adaptor protein, Grb, recruitment of Sos. Sos is a guanine nucleotide exchange factor for Ras, which leads to activation of Raf and the MAP kinases, also referred to as ERK1 and ERK2.

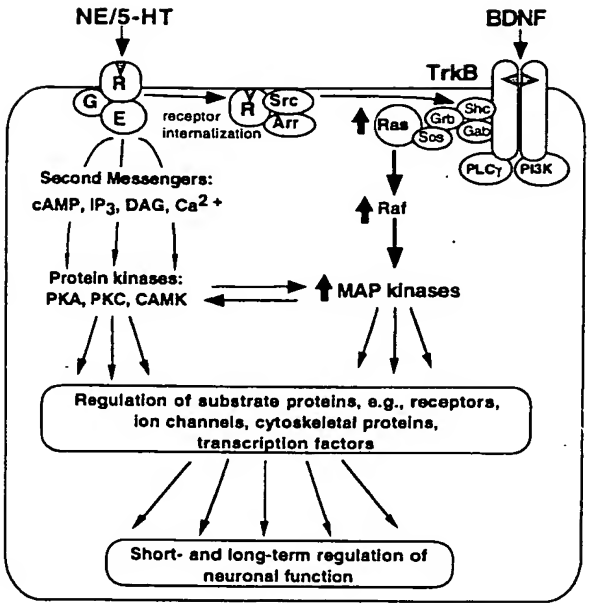

Figure 4. A model demonstrating the signal transduction pathways for neurotrophic factors. Neurotrophic factors, such as BDNF, utilize signaling pathways that are different from the second messenger-dependent systems (i.e., cAMP, IP_3, DAG, Ca^{2+}). Two molecules of BDNF bind to the TrkB receptor, inducing dimerization and tyrosine phosphorylation of the receptor itself as well as effector proteins. These include phosphatidylinositol-3 kinase (PI3K), phospholipase C-γ (PLCγ), and the MAP kinase pathway. Activation of MAP kinase occurs via several intermediate steps, including phosphorylation of Shc and Gab, then recruitment of the adapter protein Grb, and finally Sos. Sos is a guanine nucleotide exchange factor for Ras, which leads to activation of Raf and the MAP kinases. Internalization of βAR or other G protein coupled receptors can also lead to activation of the MAP kinase pathway. Internalization of the βAR is accompanied by binding of β-arresting (Arr), which acts as an adapter protein for a soluble tyrosine kinase, Src. Src is then able to substitute for TrkB and phosphorylate Shc and Gab, thereby activating the MAP kinase pathway. Antidepressant-induced internalization of βAR could lead to activation of the MAP kinase pathway, independent of the cAMP cascade.

The MAP Kinase System Can Also Be Directly Activated by Internalization of βAR and Other G Protein Coupled Receptors

In addition to the induction of BDNF expression via activation of cAMP and Ca^{2+} dependent pathways, βAR and other G protein coupled receptors are reported to activate the MAP kinases by a pathway that is independent of these second messenger systems (Figure 4) (see Luttrell

et al 1999). This alternate pathway is dependent on internalization of the βAR and recruitment of a soluble tyrosine kinase (Src) that directly phosphorylates the adaptor proteins (Shc and Gab) that lead to activation of Ras and subsequently MAP kinases. Internalization of βAR leads to binding of β-arrestin, which inhibits further G protein activation of the receptor. Recent studies demonstrate that β-arrestin also functions as an adaptor protein that binds both βAR and Src. This pathway could represent an alternate mechanism by which chronic antidepressant treatment, which results in down-regulation of βAR and other monoamine receptors, leads to activation of the MAP kinase pathway. Recent studies demonstrate that 5-HT_{1A} receptors, which are regulated by antidepressant treatment, activate the MAP kinase pathway, possibly via this mechanism (Mendez et al 1999). This could explain how 5-HT_{1A} receptors, which are negatively coupled to the cAMP system, could bypass the cAMP cascade and up-regulate the MAP kinase cascade. It should also be noted that regulation of MAP kinase via internalization of G protein coupled receptors may not be observed in all cases, indicating that there is receptor and/or cellular specificity in the control of this pathway.

Characterization of BDNF Gene Expression

The expression of BDNF in cultured cells is reported to be up-regulated by activation of the cAMP system (Nibuya et al 1995; Duman et al 1997). In addition, neuronal depolarization and activation of voltage sensitive Ca^{2+} channels are also reported to result in robust activation of BDNF expression, which could contribute to the synaptic alterations underlying the influence of BDNF on LTP. Recent studies have begun to characterize the promoter region of the BDNF gene responsible for cAMP and Ca^{2+} regulation (Shieh et al 1998; Tao et al 1998). BDNF is a complex gene of over 40 kb, which contains 5 exons. The first 4 exons contain putative promoter elements that control the expression of BDNF, and the last exon contains the entire coding region for BDNF protein. These studies demonstrate the presence of a CRE in the promoter region of exon III, which is reported to be the most highly regulated exon in response to neuronal depolarization. Activation of this element is mediated by CREB or a CREB-like protein. An additional element has also been identified that is thought to interact with CREB and thereby contribute to full and sustained activation of BDNF. The identity of this transcription factor has not been determined.

Expression of BDNF Is Up-Regulated by Antidepressant Treatment

A role for BDNF in the action of antidepressant treatment is supported by several lines of evidence. First, we have found that chronic administration of different classes of antidepressants increases the expression of BDNF in limbic brain regions, particularly the hippocampus (Nibuya et al 1995, 1996). These studies also demonstrate that antidepressant pretreatment blocks the down-regulation of BDNF in response to stress. Second, direct application of BDNF into the midbrain of rats is reported to have antidepressant effects in behavioral models of depression, including the forced swim and learned helplessness paradigms (Siuciak et al 1996). Third, BDNF is reported to be a potent neurotrophic factor for both the NE and 5-HT neurotransmitter systems (Sklair-Tavron and Nestler 1995; Mamounas et al 1995). These findings demonstrate that BDNF is a target of the cAMP system and antidepressant treatment and that BDNF is sufficient to produce an antidepressant response. Moreover, the results suggest that BDNF could influence monoamine systems via actions at either presynaptic sites (e.g., increased function of monoamine neurons) or postsynaptic sites (e.g., increased output of target neurons).

As discussed for CREB, additional studies will be required to further test the hypothesis that BDNF is an important and relevant target of antidepressant treatment. We are currently testing BDNF knock-out mice and inducible BDNF transgenic mice in neurochemical and behavioral paradigms of depression.

Summary and Conclusions

Preclinical and clinical studies of stress, depression, and action of antidepressant treatment have resulted in a molecular and cellular hypothesis of depression in which neural plasticity plays a major role (see Figure 1). These studies suggest that atrophy and death of neurons in the hippocampus, as well as prefrontal cortex and possibly other brain regions, could contribute to the pathophysiology of depression. This model also provides an explanation for individual vulnerability to stress and depression. Individual variation could result from genetic (e.g., altered expression or mutation of CREB or BDNF) or environmental factors (exposure to subtle neuronal insults, e.g., hypoxia, hypoglycemia, neurotoxins) that alone are not sufficient to cause neuronal damage and depression but that create a state of increased vulnerability to subsequent exposure to stress or other precipitating factors. Chronic antidepressant treatment, via up-regulation of CREB and BDNF, protect these neurons from further damage or possibly even reverse the atrophy and damage that has occurred. Further brain imaging and postmortem studies are needed to test this hypothesis.

The molecular mechanisms that underlie the actions of stress and antidepressant treatments are also being elucidated and could provide novel targets for therapeutic intervention. In addition to the cAMP cascade, the cellular

components of the TrkB-MAP kinase cascade could also be potential targets. The mechanisms that contribute to the actions of stress, including regulatory pathways for glucose uptake and metabolism and glutamate-induced excitotoxicity, have not been explored. It is also likely that the pathways described in this review represent only a rudimentary understanding of the intricate cellular mechanisms that underlie the etiology and treatment of depression. Continued characterization of these complex regulatory pathways and the genes they control are the long-term goals of future studies.

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Attachment 2

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Regulation of cAMP phosphodiesterase mRNAs expression in rat brain by acute and chronic fluoxetine treatment. An in situ hybridization study

Xavier Miró [a,1], Silvia Pérez-Torres [b,1], Francesc Artigas [b], Pere Puigdomènech [a], José M. Palacios [b,2], Guadalupe Mengod [b,*]

[a] *Department of Molecular Genetics, Instituto de Biologia Molecular de Barcelona, CID-CSIC. 08034 Barcelona, Spain*
[b] *Department of Neurochemistry, Institut d'Investigacions Biomèdiques de Barcelona, CSIC (IDIBAPS), 08036 Barcelona, Spain*

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Abstract

Changes in brain cyclic AMP (cAMP) have been suggested to underlie the clinical action of antidepressant treatments. Also, a regionally-selective regulation of cAMP-specific phosphodiesterases (PDEs) has been demonstrated for some antidepressants. To further investigate the effects of antidepressant treatments on PDEs, we examined the expression of different cAMP-specific PDEs in the brain of rats treated (1 and 14 days) with fluoxetine 3 mg/kg day. The mRNAs coding for PDE4A, PDE4B, PDE4D, and the five known PDE4D splice variants were analyzed by in situ hybridization on 45 brain structures of acute and chronic fluoxetine-treated rats. We also examined the binding sites for the putative antidepressant drug [^{3}H]rolipram, a PDE4-selective inhibitor. In some brain areas single fluoxetine administration increased the density of the mRNA of all PDE4 isozymes, except PDE4D and PDE4D5. Chronic fluoxetine treatment increased PDE4A mRNA levels and decreased those for PDE4B, PDE4D and PDE4D1 mRNAs in some brain regions. The study was complemented with the analysis of the expression of the transcripts of BDNF. Chronic fluoxetine treatment down-regulated the expression of BDNF. These results show that the expression of PDE4 isozymes is modulated by a clinically relevant fluoxetine dose. The significance of these changes in PDE4 expression to the antidepressant effect of fluoxetine is discussed.

Keywords: Antidepressants; In situ hybridization; Fluoxetine; Rolipram; PDE4; PDE4D splice variants; BDNF

1. Introduction

Selective serotonin reuptake inhibitors (SSRIs) are extensively used in the treatment of major depression. They exert their therapeutic effect enhancing brain serotonergic activity by preventing serotonin (5-HT) reuptake and thereby increasing its concentration in the synaptic cleft. But all antidepressant treatments require an administration of at least two weeks before inducing a clinically significant improvement. The mechanisms underlying in this delay in the therapeutic effect of SSRIs remain unknown.

Cyclic AMP (cAMP) plays an important role in signal transduction processes (Houslay, 1998). A role of the cAMP pathway has been suggested in several CNS diseases such as depression (Duman et al., 1997) and Alzheimer's disease (Cowburn et al., 1996; Bonkale et al., 1999). The hydrolysis of cAMP is regulated by a family of cyclic nucleotide phosphodiesterases (PDEs). Twelve members of this family have been identified until now on the basis of their substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and amino acid sequences (Conti and Jin, 1999; Soderling and Beavo, 2000; Houslay, 2001). Families 4, 7 and 8 specifically hydrolyze cAMP. The PDE4 family has four members (PDE4A through PDE4D) (Houslay, 1998), the PDE7 family, two (PDE7A, PDE7B) (Conti

* Corresponding author. Tel.: +34-93-363-83-23; fax: +34-93-363-83-01.
E-mail address: gmlnqr@iibb.csic.es (G. Mengod).
[1] These two authors have contributed equally to the work.
[2] Permanent address: Research Center, Almirall Prodesfarma S.A., Cardener 68-74, E-08024 Barcelona, Spain.

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and Jin, 1999) and the PDE8 two (PDE8A, PDE8B) (Fisher et al., 1998; Hayashi et al., 1998) encoded by different genes.

The main objective of the present study was to examine the putative involvement of PDE4s in the mechanism of action of antidepressant drugs. For this, we analyzed the effects of single and repeated fluoxetine treatment on the regulation of the expression of different cAMP-specific PDE4s in a large number of brain structures. The study was complemented by the analysis of the expression of BDNF and [^{3}H]rolipram binding on the same brain structures.

2. Materials and methods

2.1. Animals and treatments

Adult male Wistar rats (200–300 g) were purchased from Iffa Credo (Lyon, France). Animals were acclimated to standard laboratory conditions (14-h light/10-h dark cycle) with free access to rat chow and water. Each rat was used only once for experimentation, and all the procedures conformed to the European Communities Council directive of November 24, 1986 (86/609/EEC). Animals were conscious and freely moving at all times throughout the experimental procedure. Rats were randomly assigned to receive vehicle or fluoxetine. In the case of acute treatments, rats were administered i.p. with fluoxetine (3 mg/kg) or vehicle and killed by decapitation 2 h later. For the chronic treatments, rats under light ether anesthesia were implanted s.c. with Alzet 2002 minipumps filled to deliver vehicle (water/DMSO 50%/50%) or fluoxetine dissolved in vehicle (3 mg/kg day) for 14 days. Animals were killed by decapitation with the minipumps on board. These were removed to check that they were empty by visual inspection. The use of dimethyl sulfoxide (DMSO) to dissolve fluoxetine was necessary due to the small volume of the minipumps. Given the weight gain of the animals, the doses used correspond to the 7th day of treatment. The animals were killed by decapitation without using anesthesia to prevent potential interferences on the variables measured. Brains were quickly removed from the skull, frozen on dry ice and kept at −20°C. Tissue sections, 14-μm thick, were cut on a microtome–cryostat (Microm HM500 OM, Waldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma, St. Louis, MO) and kept at −20°C until used.

2.2. In situ hybridization histochemistry

The oligonucleotides used were complementary to the following base sequences (Acc. no. inside the brackets): PDE4A 3649–3693 (L27057), PDE4B 2639–2687 (U95748), PDE4D, bases 1586–1630 (U09456); PDE4D1, bases 180–219 (U09455); PDE4D2, bases 117–163 (U09456); PDE4D3, bases 1–45 (U09457); PDE4D4, bases 360–404 (AF031373); PDE4D5, bases 251–295 (AF012073). They were synthesized by Amersham Pharmacia Biotech (Little Chalfont, UK). The oligonucleotide used for BDNF was the same as described previously (Rocamora et al., 1992).

The oligonucleotides were labeled at their 3′-end by using [α-^{32}P]dATP (3000 Ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyltransferase (TdT, Roche Molecular Biochemicals, Mannheim, Germany) to specific activities of 0.7–1.2×10^4 Ci/mmol. Labeled probes were purified by QIAquick Nucleotide Removal Kit (QIAGEN, Hilden, Germany).

Prior to hybridization frozen tissues were brought to room temperature, air dried and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4), washed once in 3×PBS, twice in 1×PBS, 5 min each, and incubated in a freshly prepared solution of predigested pronase at a final concentration of 24 U/ml in 50 mM Tris–HCl pH 7.5, 5 mM EDTA for 2 min at room temperature. Proteolytic activity was stopped by immersion for 30 s in 2 mg/ml glycine in PBS. Tissues were rinsed in PBS and dehydrated in a graded series of ethanol 100%. For hybridization, labeled probes were diluted to a final concentration of approximately 10^4 cpm/μl in a solution containing 50% formamide, 4×SSC, 1×Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 20 mM phosphate buffer pH 7, 250 μg/ml yeast tRNA, and 500 μg/ml salmon sperm DNA. Tissues were covered with 100 μl of the hybridization solution and overlaid with Nescofilm coverslips to prevent evaporation. Tissues were incubated in humid boxes overnight at 42°C and then washed 4 times (45 min

each) in 600 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA at 60°C. Hybridized sections were exposed to Hyperfilm ß-max (Amersham) for 3–5 weeks at −70°C with intensifying screens.

2.3. Autoradiography

Autoradiography with [^{3}H]-rolipram was performed as previously described (Kaulen et al., 1989; Perez-Torres et al., 2000). [^{3}H]Rolipram (85 Ci/mmol) was purchased from Amersham (Little Chalfont, UK). (±)-Rolipram was a generous gift from Almirall Prodesfarma (Barcelona, Spain). Tissue sections were preincubated for 15 min at room temperature in 150 mM phosphate buffer pH 7.4, containing 2 mM $MgCl_2$ and 100 µM dithiothreitol. They were then incubated for 1 h at 0°C in the same buffer with [^{3}H]rolipram 2 nM. Adjacent sections were incubated in the presence of 1 µM (±)-rolipram to determine the non-specific binding. After incubation, the sections were washed twice for 5 min each in the same buffer at 0°C, dipped in distilled water at 0°C and rapidly dried under a cold air stream (4°C). Autoradiograms were generated by apposing the labeled tissue sections to a Hyperfilm ^{3}H (Amersham) together with plastic ^{3}H-standards (^{3}H-Microscales, Amersham) in X-ray cassettes for 4 weeks at 4°C.

To analyze the degree of blockade of the 5-HT transporter by acute and chronic fluoxetine treatments, we carried out an additional autoradiographic study using [^{3}H]citalopram as a ligand. Tissue sections were preincubated for 15 min at room temperature in a buffer containing 120 mM NaCl, 5 mM KCl, and 50 mM Tris–HCl (pH 7.4). Additional experiments were conducted using a much longer (4 h) preincubation time. The reason to use two different preincubation times was the following. Differences in [^{3}H]citalopram binding between control and treated rats could theoretically be due to the presence of the drug in brain or (less likely) to fluoxetine-induced changes in the density of the 5-HT transporter. To examine the second possibility, a long preincubation time was additionally used to wash away the drug remaining in the tissue sections. Thereafter, sections were incubated for 1 h at room temperature in the same buffer with 1 nM [^{3}H]citalopram (79.5 Ci/mmol, NEN, Boston, USA). Adjacent sections were incubated in the presence of 1 µM imipramine (RBI, Natick, USA), to determine non-specific binding. Sections were washed 2×10 min in the same buffer at 4°C, dipped in distilled water at 4°C and dried rapidly under a cold air stream (4°C). Autoradiograms were generated by apposing the labeled tissue sections to a ^{3}H-Hyperfilm (Amersham) together with plastic ^{3}H-standards (^{3}H-Microscales, Amersham) in X-ray cassettes for 6 weeks at 4°C. Single and repeated treatment with fluoxetine elicited a dramatic reduction of the binding of [^{3}H]citalopram in brain compared to controls, as assessed by autoradiography using standard experimental conditions (15 min preincubation time). The use of a much longer preincubation time (4 h) resulted in a recovery of the autoradiographic signal to values similar to those in controls. The binding of [^{3}H]citalopram in controls to the 5-HT transporter was unaffected by the length of the preincubation period (data not shown). These observations support the notion that the dose of fluoxetine used (3 mg/kg, single or continuously for 14 days) elicited a significant blockade of the 5-HT transporter.

2.4. Data analyses

Quantitative image analysis was performed with the MCID-4 computerized image analysis system (St Catharines, Ontario, Canada) on 45 brain regions.

Quantitative measures were analyzed using two-way ANOVA (SigmaStat, Jandel Co, Chicago, IL) analyses for each PDE isozyme, BDNF and [^{3}H]rolipram binding using the regions in which the effect of fluoxetine treatment (single or chronic) differed ±10% from control values. These regions were analyzed considering the effect of fluoxetine treatment (vehicle and treated), the anatomical region, and treatment-region interaction. The latter assesses whether the possible effect of fluoxetine treatment is different for the different regions considered. Since the anatomical region factor was statistically significant for all variables examined (PDE4s, BDNF and [^{3}H]rolipram binding), we only give the p values corresponding to the treatment factor and/or the treatment×region interaction, both being representative of a significant effect of the treatments. Significance level has been set at $p<0.05$ (two-sided). Post hoc analysis (Tukey's test) was performed with a significance set at $p<0.05$ when ANOVA indicated that the treatment factor and/or treatment×region interaction was significant ($p<0.05$). In the Figs 2–5, asterisks (*) were placed only when ANOVA and Tukey's post test indicated a significant treatment×region interaction ($p<0.05$).

3. Results

We have examined the effects of the acute and chronic treatment with the selective serotonin reuptake inhibitor fluoxetine on the expression of the four members of the PDE4 family (PDE4A, PDE4B, PDE4C and PDE4D, including its five mRNA splice variants), and BDNF mRNAs, by in situ hybridization histochemistry. We have also analyzed the effects of fluoxetine treatments on the density of [^{3}H]rolipram binding sites by autoradiography. Figure 1 shows representative autoradiograms of rat coronal sections of a similar level from control (A1–J1), acute (A2–J2), and chronic (A3–J3) fluoxetine treatment from [^{3}H]rolipram binding sites (A1–A3) and in situ hybridization for mRNAs coding for PDE4A

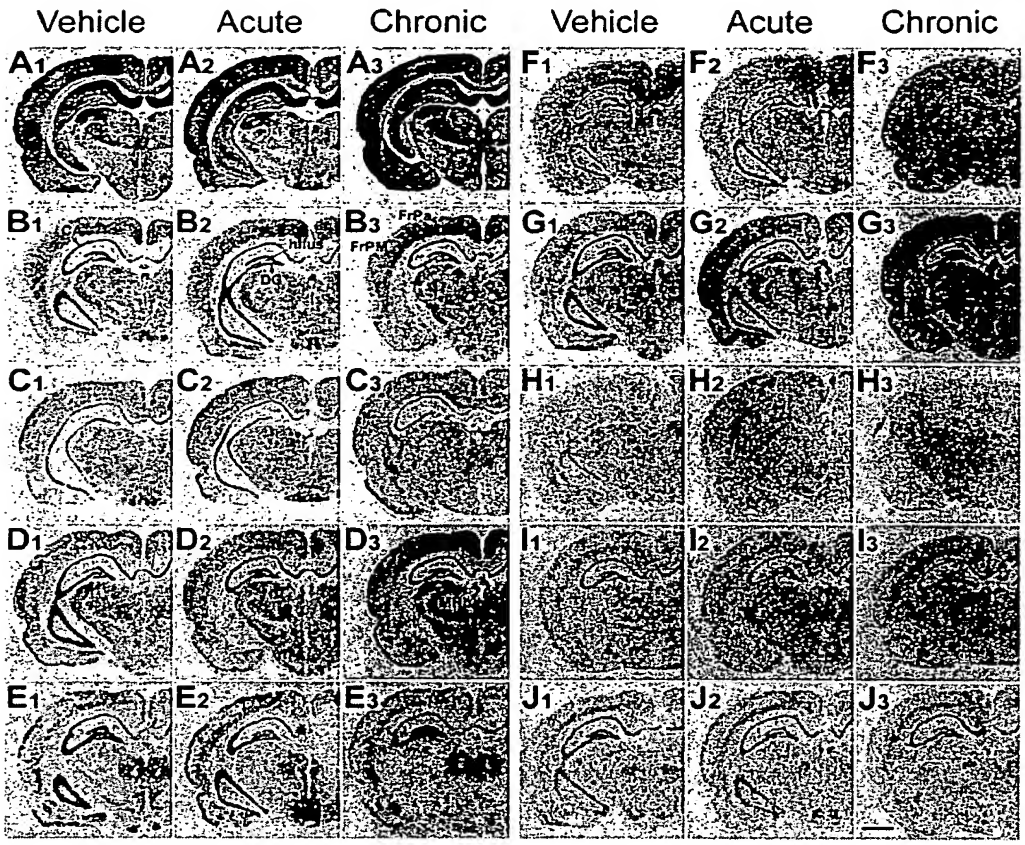

Fig. 1. Rat coronal brain sections from control (A1–J1), acute (A2—J2), and chronic (A3–J3) fluoxetine treated. Rats were treated as described in Material and Methods. Film autoradiogram pictures from [^{3}H]rolipram incubation (A1–A3), and in situ hybridization for mRNAs coding for PDE4A (B1–B3), PDE4B (C1–C3), PDE4D (D1–D3), BDNF (E1–E3), and PDE4D splice variants PDE4D1 (F1–F3), PDE4D2 (G1–G3), PDE4D3 (H1–H3), PDE4D4 (I1–I3), PDE4D5 (J1–J3). Frontoparietal cortex (FrPa, FrPm), anterior cingulate cortex (ACg), Ammon's horn (CA), dentate gyrus (DG), hilus, medial habenular nucleus (MHb). Bar=2 mm.

(B1–B3), PDE4B (C1–C3), PDE4D (D1–D3), BDNF (E1–E3), and PDE4D splice variants PDE4D1 (F1–F3), PDE4D2 (G1–G3), PDE4D3 (H1–H3), PDE4D4 (I1–I3), PDE4D5 (J1–J3). The results of the optical density measurements obtained are summarized in Figs 2 to 5.

Due to the fact that PDE4C mRNA could be detected exclusively in the anterior part of the main olfactory bulb, as we have previously described (Perez-Torres et al., 2000), this PDE4 isozyme was not included in the present work.

3.1. Regulation of PDE4 isoforms and BDNF mRNAs expression by antidepressant treatments

The density of [^{3}H]rolipram binding sites increased after single fluoxetine treatment in the frontal cortex, frontoparietal cortex, anterior cingulate cortex, caudate-putamen, parabigeminal nucleus, inferior olive and in white matter tracts such as corpus callosum. In contrast, an important reduction in the area postrema (Fig. 2A) could be observed. Two-way ANOVA revealed a significant effect of the treatment ($F_{1,54}$=6.32; p=0.015; Tukey's post hoc test $p<0.05$). Conversely, long-term fluoxetine treatment decreased [^{3}H]rolipram binding sites in these same regions except in the parabigeminal nucleus, inferior olive and area postrema (Fig. 4A). However, an increase in [^{3}H]rolipram binding sites was found in the hilus, medial habenular nucleus, medial geniculate nucleus, and pontine nuclei (region×treatment interaction effect; $F_{8,54}$=2.83; p=0.01, Tukey's post hoc test $p<0.05$) where no change in binding was found following the single injection of fluoxetine, while levels in the dorsal part of the medial geniculate nucleus were increased by both treatments.

In the case of PDE4A, acute fluoxetine treatment increased its mRNA levels in only three structures: parabigeminal nucleus, vestibular nuclei and cerebellum (treatment effect; $F_{1,18}$=8.14; p=0.01; Tukey's post hoc test $p<0.05$) (Fig. 2B). Chronic fluoxetine treatment increased PDE4A mRNA levels in dentate gyrus, hilus and cerebellum; but they were significantly reduced (treatment×region interaction; $F_{5,36}$=3.01; p=0.022) in

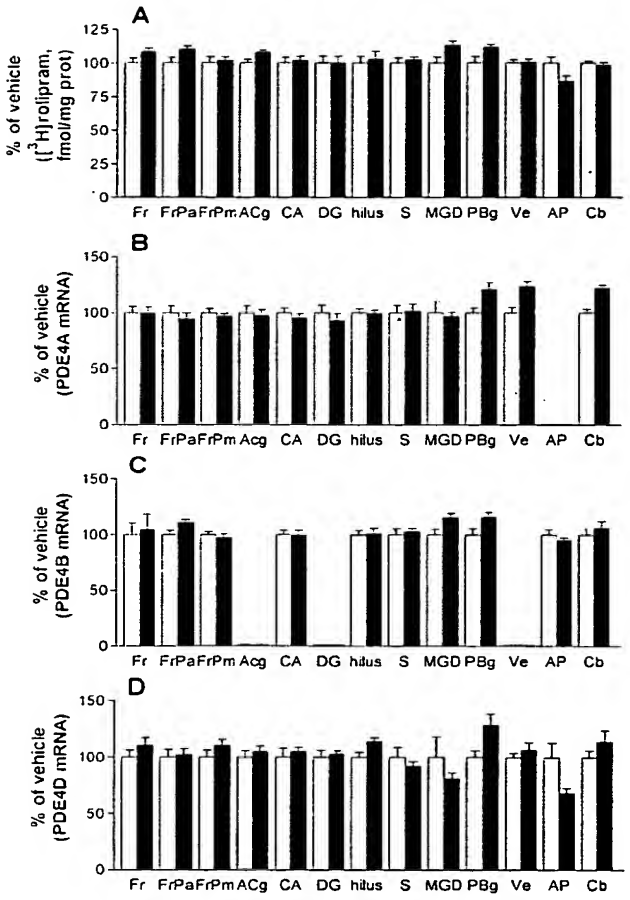

Fig. 2. Effects of acute fluoxetine treatment on the binding of [^{3}H]rolipram (A) and the expression of PDE4A (B), PDE4B (C), PDE4D (D) mRNAs in rat brain. Binding density and mRNA expression levels were determined by receptor autoradiography and in situ hybridization histochemistry analyses as those shown in Fig. 1. The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis revealed a significant effect of the treatment or treatmentxregion interaction for [^{3}H]rolipram binding, all PDEs except PDE4D (see Results for more detailed statistical analysis). Asterisks (*) indicate a significant treatmentxregion interaction, *p<0.05 compared with control (Two-way ANOVA and Tukey's post hoc test). Open bars represent control values and filled bars represent values from treated rats. Abbreviations: frontal cortex (Fr), frontoparietal cortex (FrPa, FrPm), anterior cingulate cortex (ACg), Ammon's horn (CA), dentate gyrus (DG), hilus, subiculum (S), dorsal part of medial geniculate nucleus (MGD), parabigeminal nucleus (PBg), pontine nuclei (Pn), vestibular nuclei (Ve), area postrema (AP), cerebellum (Cb).

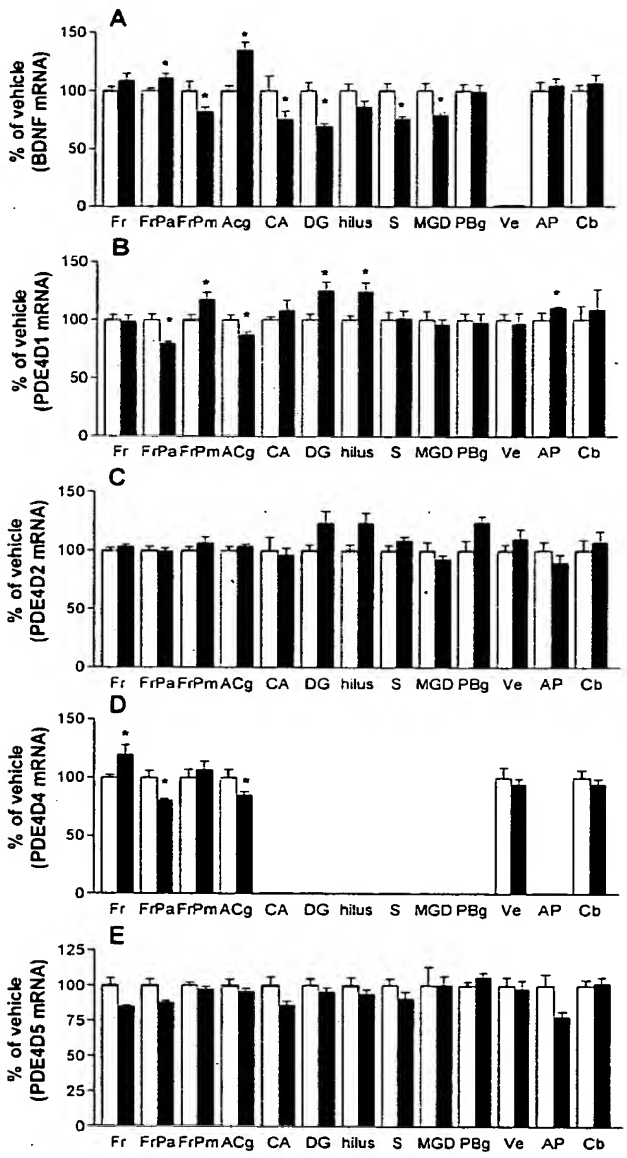

Fig. 3. Bar chart showing the effect of acute fluoxetine treatment on the expression of mRNAs coding for BDNF (A), PDE4D1 (B), PDE4D2 (C), PDE4D4 (D), and PDE4D5 (E). The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis showed a significant effect of the treatment or treatmentxregion interaction for PDE4D splicing isoforms. Asterisks (*) indicate a significant treatmentxregion interaction, *p<0.05 compared with control (Two-way ANOVA and Tukey's post hoc test). Levels of PDE4D3 mRNA were not significantly altered by the acute treatment (data not shown). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

cingular and frontoparietal cortices, and medial septal nucleus. Multiple comparison Tukey's post hoc test showed a significant interaction of the treatment in cortical and hipocampal regions (p<0.05) (Fig. 4B).

Acute fluoxetine treatment increased PDE4B mRNA levels in seven brain structures: frontoparietal cortex, caudate-putamen, medial habenular nucleus, dorsal part of the medial geniculate nucleus, parabigeminal nucleus, inferior olive and white matter tracts such as corpus callosum (treatment effect $F_{1,42}$=21.84; p=0.0003; Tukey's post hoc analysis p<0.05) (Fig. 2C). Conversely, a reduction in PDE4B mRNA expression was measured

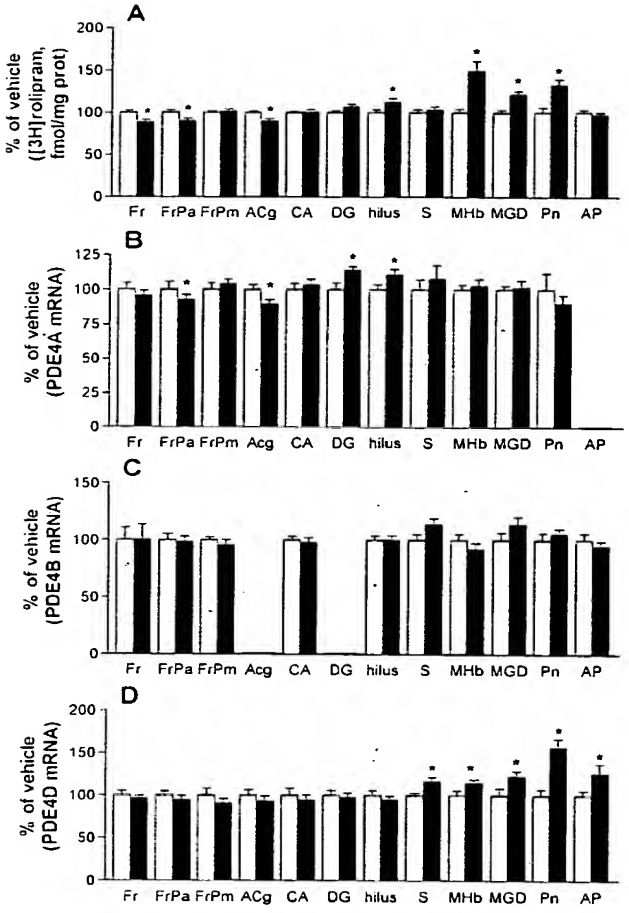

Fig. 4. Influence of chronic fluoxetine treatment on the binding of [^{3}H]rolipram (A) and the expression of PDE4A (B), PDE4B (C), and PDE4D (D) mRNAs. Binding density and mRNA expression levels were determined by receptor autoradiography and in situ hybridization histochemistry analyses as those shown in Fig. 1 and quantified by densitometry as described in Materials and Methods. The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis revealed a significant effect of the treatment or treatmentxregion interaction for [^{3}H]rolipram binding, all PDEs (see Results for more detailed statistical analysis). Asterisks (*) indicate a significant treatmentxregion interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

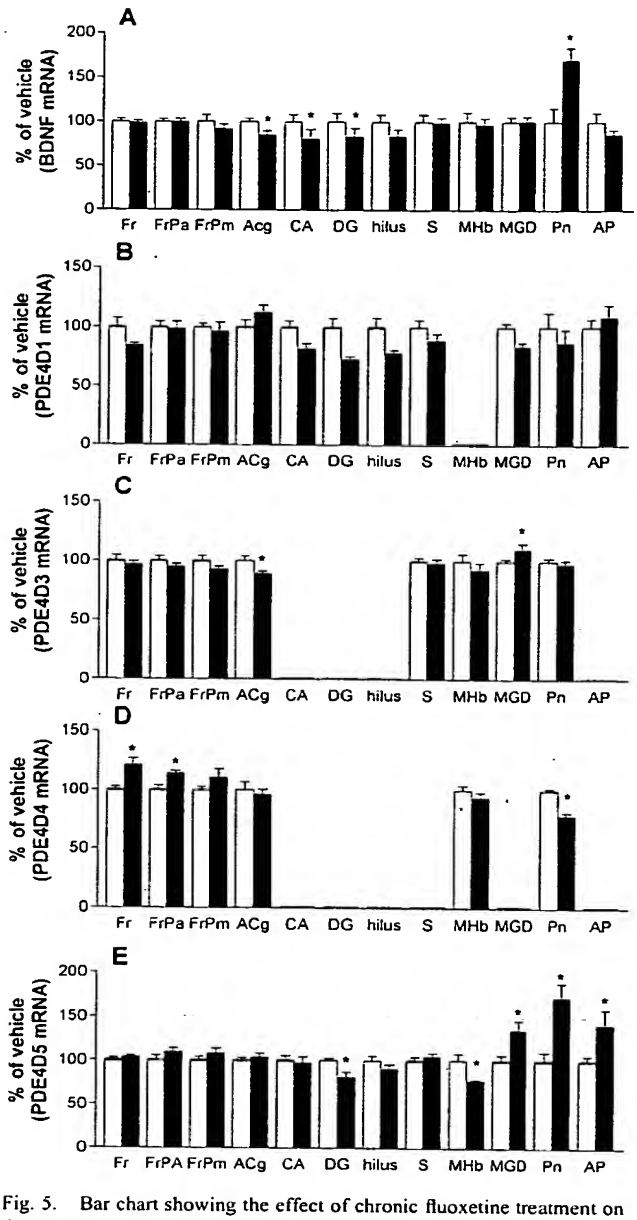

Fig. 5. Bar chart showing the effect of chronic fluoxetine treatment on the expression of mRNAs coding for BDNF (A), PDE4D1 (B), PDE4D3 (C), PDE4D4 (D), and PDE4D5 (E). The results are expressed as mean ±SEM percent of control; n=4 per group. Two-way ANOVA analysis showed a significant effect of the treatment×region interaction for PDE4D splicing isoforms. Asterisks (*) indicate a significant treatmentxregion interaction, $*p<0.05$ compared with control (Two-way ANOVA and Tukey's post hoc test). Levels of PDE4D2 mRNA were not significantly altered by the chronic treatment (data not shown). Open bars represent control values and filled bars represent values from treated rats. Abbreviations are as in Fig. 2.

after chronic fluoxetine treatment in caudate-putamen, inferior olive, cerebellum, pineal gland and corpus callosum and a significant increase (treatment effect; $F_{1,42}$=5.96; p=0.019; Tukey's post hoc test $p<0.05$) (Fig. 4C) in subiculum and medial geniculate nucleus.

PDE4D mRNA transcripts were increased after single fluoxetine treatment in frontal cortex, hilus, medial habenular nucleus and parabigeminal nucleus (treatment effect: $F_{1,24}$=7.07; p=0.014; Tukey's post hoc test $p<0.05$). These mRNA levels were decreased in the dor-

sal part of the medial geniculate nucleus and in the area postrema (Fig. 2D). However, these latest changes did not attain statistical significance (treatment effect; $F_{1,12}$=2.3; p=0.15). In contrast, chronic fluoxetine treatment significantly increased PDE4D mRNA expression in subiculum, medial habenular nucleus, medial geniculate nucleus, pontine nuclei, and area postrema (treatment×region interaction; $F_{6,42}$=5.16; p=0.0005). Multiple comparison Tukey's post hoc test revealed a significant difference (p<0.05) between control and treatment group in these regions (Fig. 4D).

Acute fluoxetine treatment resulted in a reduction of BDNF transcripts in the Ammon's horn, dentate gyrus, subiculum and dorsal part of medial geniculate nucleus and in the posterior part of frontoparietal cortex but in an increase in frontoparietal and anterior cingulate cortices and inferior olive (treatment×region interaction; $F_{6,42}$=3.42; p=0.0077; Tukey's post hoc test p<0.05) (Fig. 3A). After 14-day treatment, BDNF mRNA levels decreased in the Ammon's horn, dentate gyrus, anterior cingulate cortex, parabigeminal nucleus and inferior olive. In contrast, high BDNF mRNA levels were detected in the pontine nuclei. Two-way ANOVA and Tukey's post hoc test revealed a significant treatment×region interaction in these regions. ($F_{5,36}$=6.17; p=0.00032; Tukey's post hoc test p<0.05) (Fig. 5A).

3.2. Regulation of PDE4D splice variants mRNA by antidepressant treatments

We extended our study to the expression of the five PDE4D splice variants in the same brain structures. The hybridization conditions and differential anatomical localization in the rat brain has been previously described (Miro et al., 2002). Each PDE4D splice variant was differentially affected by acute and chronic fluoxetine treatments. A single fluoxetine dose increased PDE4D1 mRNA transcript levels in the posterior part of frontoparietal cortex, lateral septal nucleus, dentate gyrus, hilus and area postrema whereas they were decreased in the anterior cingulate cortex and anterior part of frontoparietal cortex (treatment effect; $F_{1,36}$=6.02; p=0.019 and treatment×region interaction; $F_{5,36}$=5.3; p=0.0009) (Fig. 3B). Multiple comparison Tukey's post hoc analysis showed a significant effect of the treatment (p<0.05). Long-term fluoxetine treatment reduced PDE4D1 mRNA levels in frontal cortex, Ammon's horn, dentate gyrus, hilus, dorsal part of medial geniculate nucleus and parabigeminal nucleus (treatment effect; $F_{1,30}$=17.16; p=0.00026; Tukey's post hoc test p<0.05) (Fig. 5B).

The mRNA coding for PDE4D2 increased after acute but not chronic treatment. A significant effect of a single fluoxetine administration was observed in the dentate gyrus, hilus, medial habenular nucleus and parabigeminal nucleus (treatment effect; $F_{1,24}$=17.14; p=0.0004; Tukey's post hoc test p<0.05) (Fig. 3C). The effects did not reach statistical significance in the chronically-treated group. Conversely, the chronic (but not acute) fluoxetine treatment reduced the expression of PDE4D3 mRNA splice variant in the anterior cingulate cortex and increased it in the dorsal part of medial geniculate nucleus (treatment×region interaction; $F_{1,12}$=5.55; p=0.036). Tukey's post hoc test analysis showed a significant interaction of the treatment in both regions (Fig. 5C).

In the case of PDE4D4 mRNA splicing form, acute fluoxetine treatment increased mRNA levels in frontal cortex and decreased them in frontoparietal cortex and medial septal nucleus (treatment×region interaction; $F_{2,18}$=4.69; p=0.023; Tukey's post hoc test p<0.05) (Fig. 3D). On the other hand, chronic treatment resulted in an increment of PDE4D4 mRNA hybridization signal in the frontal and frontoparietal cortex (Fig. 5D) and in a reduction in pontine, vestibular and cerebellar nuclei (treatment×region interaction; $F_{4,30}$=8.6; p=0.00009; Tukey's post hoc test p<0.05).

Hybridization signal intensity for PDE4D5 mRNA decreased in acute fluoxetine treated rats in the frontal and frontoparietal cortices, Ammon's horn, and area postrema (treatment effect; $F_{1,24}$=7.04; p=0.014; Tukey's post hoc test: p<0.05) (Fig. 3E). A reduction was also observed after chronic fluoxetine treatment in the dentate gyrus and medial habenular nucleus, whereas an increment was detected in the medial geniculate nucleus, pontine nucleus and area postrema (treatment effect; $F_{1,30}$=4.36; p=0.045; and treatment×region interaction; $F_{4,30}$=6.54; p=0.0006). Multiple comparison Tukey's post hoc test showed a significant difference between control and treated group in these regions; p<0.05 (Fig. 5E).

4. Discussion

The aim of the present work was to study the alterations on the transcription levels of the mRNAs coding for PDE4A, PDE4B, PDE4D and its five splice forms, in the rat brain upon acute and chronic treatment with a 5-HT transporter inhibitor, fluoxetine. When studying the correlation between the changes in the levels of both ^{3}H-rolipram binding sites and mRNA coding for each of the PDE4 isozymes analyzed, several considerations should be taken into account: (i) ^{3}H-rolipram labels only the high affinity site present in the PDE4 isozymes, but it does not label their catalytic site. (ii) In the brain, there is an anatomical component that makes it difficult to establish unequivocal correlations between mRNA and protein content, in a given area, due to its complex architecture. A coincidence in the changes observed for both parameters in a particular brain area would be consistent

with their somatodendritic localization, whereas a non-coincident alteration could be due to a location of the isozymes in neuronal terminals, far away from the cell bodies that contain the mRNA. (iii) The relationship between the transcription and the translation of the mRNA coding for the different PDE4 isozymes is not yet known in rat brain and it may be complex.

A role for the cAMP cascade in the long-term actions of antidepressant treatments has been suggested (Menkes et al., 1983; Nestler et al., 1989; Nibuya et al., 1996). The cortical and hippocampal cAMP systems are potential common targets for 5-HT and noradrenaline actions given the innervation of these territories by both transmitters and the convergence of intracellular signals resulting from the activation of adenylyl cyclase-coupled receptors (Duman et al., 1997). Indeed, rolipram and other family-selective PDE inhibitors (O'Donnell and Frith, 1999) display antidepressant properties.

Our data indicate that the different PDE4s are distinctly regulated by acute and chronic fluoxetine treatment. Acute fluoxetine treatment increased significantly [^{3}H]rolipram binding and the expression of the PDE4 family members, with the exception of PDE4D. This increase could result from an increase of cAMP levels (Duman et al., 1997) after the additional tone on 5-HT receptors positively coupled to adenylyl cyclase. Consistently, activation of the cAMP pathway increased the expression of PDE4 family members (Houslay, 1998). The unchanged expression of PDE4D appears to result from opposite changes in PDE4D2 (increase) and PDE4D5 (decrease) whereas the effects on PDE4D1 and PDE4D4 depend on the structure examined. In cultured cells, the activation of the cAMP system increased the expression of short PDE4D forms (PDE4D1 and PDE4D2) (Swinnen et al., 1989). Thus, elevated cAMP levels could explain the increase in PDE4D1 and PDE4D2 in the hippocampus.

Acute fluoxetine administration increases the expression of BDNF in cortex and the inferior olive while it decreases in other areas, including the hippocampus (CA fields and dentate gyrus). A trend to decreased BDNF mRNA levels (1.8 kb transcript by Northern blot analysis) in cortex and hippocampus was also observed after single treatment with sertraline (Nibuya et al., 1995).

Two-week fluoxetine treatment altered PDE4 mRNA expression in a manner different from acute treatment. Given the progressive and delayed onset of clinical antidepressant action, long-term changes may be related to the therapeutic effect of fluoxetine. Thus, changes in the mRNAs of PDE4 family members in several brain regions, could lead to an increase in cAMP accumulation with putative therapeutic benefits. Thus, a down-regulation of [^{3}H]rolipram binding sites is detected in corpus callosum, caudate-putamen, and several cortical areas. PDE4B mRNA decreases in the cerebellum (contrary to PDE4A), caudate-putamen, corpus callosum, pineal gland and inferior olive. PDE4A mRNA also decreases in the septum, anterior cingulate cortex and frontoparietal cortex. These results are in disagreement with the report by Takahashi et al. (1999) showing up-regulation of PDE4A and PDE4B mRNA in frontal cortex, and PDE4B mRNA in nucleus accumbens after chronic sertraline and fluoxetine (5 mg/kg day, 21 days). An increased PDE4B transcription in frontal cortex (but not hippocampus) was reported (Suda et al., 1998) after 21-day imipramine treatment. However, and in agreement with our observations, an increased hippocampal expression of PDE4A (no change in frontal cortex) has been reported after 2-week treatment with 5 mg/kg day fluoxetine (Ye et al., 2000).

Chronic treatment with fluoxetine resulted in a decrease of PDE4D expression in vestibular and cerebellar nuclei, and cerebellum, and in an increment in several other brain areas. Reductions in PDE4D1 mRNA levels are observed in six brain areas, including hippocampal CA fields and dentate gyrus (in common with PDE4D5). A marked increase in PDE4D mRNA (through the PDE4D5 isoform) occurs in the pontine nuclei. This area plays a key role in the regulation of sleep via cAMP pathway (Capece and Lydic, 1997). The increase in pontine PDE4D5 mRNA could be related to the long-term effects of antidepressants on sleep architecture, in particular the reduction of REM sleep (Sharpley and Cowen, 1995; Zajecka, 2000). Indeed, the pontine nuclei play a major role in the generation of REM sleep which is associated with increased (ACh) and decreased (5-HT and NE) neuronal activity (Jones, 1991).

Contrary to our expectations, the levels of BDNF mRNA decreased after chronic fluoxetine treatment in the hippocampus (DG and CA), increasing only in the pontine nuclei. Chronic antidepressant treatment was reported to augment CREB and BDNF expression in hippocampus (Nibuya et al., 1996). This led to the suggestion that the cAMP cascade may be a common cellular pathway for antidepressant drug action (Duman et al., 1997). However, 21-day treatments with two selective PDE4 inhibitors (rolipram and Ro 20-1724) or NE reuptake blockers (desipramine and Org, 4428) did not increase the expression of BDNF mRNA. This was only enhanced by combinations of the PDE4 inhibitor and NE reuptake inhibitors (Fujimaki et al., 2000). A closer inspection of the data by Nibuya et al. (1995) showed a moderate 20% increase of the 4.4. kb transcript (less abundant form in hippocampus (Hofer et al., 1990) after 21-day sertraline treatment.

The reasons for the discrepancy between our study and that of Nibuya et al. (1996) are unclear and perhaps involve methodological reasons. Indeed, in situ densitometric mRNA measurements done in brain areas are more accurate than those obtained after dissecting and homogenizing hippocampal tissue. Moreover, our stat-

istical analysis was done with a single averaged value per rat whereas Nibuya et al. (1996) used replicate measures per rat. In the present study fluoxetine was delivered by minipumps to prevent the injection-induced stress whereas Nibuya et al. (1996) administered the drugs i.p. Differences in the fluoxetine doses in both cases could also explain the discrepancy observed on BDNF changes. The dose used in the present study was intended to mimic the effects of the standard clinical dose since it produced a substantial increment (300% of controls) in cortical extracellular 5-HT after 14-day but not after single treatment (Hervas et al., 2001). The present data indicate that this dose elicits a substantial occupancy of the 5-HT transporter, as assessed by receptor autoradiography. Altogether, these data suggest that this fluoxetine dose was sufficient to increase the serotonergic function after repeated, but not single dosage, in parallel with its clinical effects.

The reduction of hippocampal BDNF expression is consistent with an increased tonic activation of hippocampal 5-HT_{1A} receptors produced by chronic antidepressant treatments (Haddjeri et al., 1998). 5-HT_{1A} receptors are very densely expressed in the hippocampus (Pompeiano et al., 1992) and are negatively coupled to adenylyl cyclase (De Vivo and Maayani, 1986). Thus, the antidepressant-induced activation of hippocampal postsynaptic 5-HT_{1A} receptors is more likely to reduce the hippocampal cAMP pathway (and possibly in BDNF expression) than the opposite. Also, since 5-HT_{1A} receptors are also coupled to a hyperpolarizing GIRK channel (Andrade et al., 1986) the additional activation of 5-HT_{1A} receptors during chronic fluoxetine argues against a CREB-mediated BDNF increase since CREB is activated by cellular depolarization (Sheng and Greenberg, 1990). Moreover, antidepressant drugs (including fluoxetine) have a direct inhibitory action on CRE-directed gene transcription by interfering with the above mechanism (Schwaninger et al., 1995). Furthermore, mechanisms other than the cAMP pathway can regulate BDNF mRNA expression since alternative usage of four promoters within the BDNF gene has been reported (Timmusk et al., 1993).

The results demonstrate that a clinically relevant dose of fluoxetine differentially modulates the expression of the cAMP-specific PDE4 isoenzymes, suggesting that these changes in expression may mediate the effects of antidepressant treatment.

Acknowledgements

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Attachment 3

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The effects of compounds varying in selectivity as 5-HT_{1A} receptor antagonists in three rat models of anxiety

Guy Griebel [a,*], R. John Rodgers [b], Ghislaine Perrault [a], David J. Sanger [a]

[a] *Sanofi-Synthélabo, 31 Avenue Paul Vaillant-Couturier, 92220 Bagneux, France*
[b] *School of Psychology, University of Leeds, Leeds LS2 9JT, UK*

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Abstract

Compounds varying in selectivity as 5-HT_{1A} receptor antagonists have recently been reported to produce benzodiazepine-like antianxiety effects in mice. To assess the cross-species generality of these findings, the present experiments compared the effects of diazepam (0.625–5 mg/kg) with those of several non-selective (MM-77, 0.03–1 mg/kg and pindobind-5-HT_{1A}, 0.1–5 mg/kg) and selective (WAY100635, 0.01–10 mg/kg, *p*-MPPI, 0.01–3 mg/kg and SL88.0338, 0.3–10 mg/kg) 5-HT_{1A} receptor antagonists in three well-validated anxiolytic screening tests in rats: punished lever-pressing, punished drinking, and the elevated plus-maze. In the punished lever-pressing conflict test, none of the 5-HT_{1A} receptor antagonists modified rates of punished responding, whereas in the punished drinking test, WAY100635 (0.3–1 mg/kg), SL88.0338 (3–10 mg/kg), *p*-MPPI (1 mg/kg), MM-77 (0.03–0.3 mg/kg), but not pindobind-5-HT_{1A}, produced clear anticonflict activity. However, the increase in punished responding with the 5-HT_{1A} compounds was smaller than that produced by diazepam, indicating weaker anxiolytic-like activity. In the elevated plus-maze test, WAY100635 (0.1–0.3 mg/kg), SL88.0338 (0.3–10 mg/kg), MM-77 (0.01–3 mg/kg), pindobind-5-HT_{1A} (0.1–3 mg/kg), but not *p*-MPPI, showed anxiolytic-like activity on traditional behavioral indices, increasing the percentage of time spent in open arms and the percentage of open arm entries. As was the case in the punished drinking test, the magnitude of the positive effects of the 5-HT_{1A} compounds was generally smaller than that of diazepam. Of the ethological measures recorded in the plus-maze, all compounds markedly decreased risk assessment (i.e. attempts) over the entire dose-range, but only diazepam clearly increased directed exploration (i.e. head-dipping). Although the present results demonstrate that 5-HT_{1A} receptor antagonists elicit anxiolytic-like effects in rats, this action appears to be test-specific and, unlike previous findings in mice, smaller than that observed with benzodiazepines. The data are discussed in relation to the possible relevance of species differences in 5-HT_{1A} receptor function and the nature of the anxiety response studied.

Keywords: 5-HT_{1A} receptor antagonists; Anxiety; Conflict tests; Diazepam; Elevated plus-maze; Rats

1. Introduction

After more than three decades of preclinical research on the relationship between serotonin (5-HT) and anxiety, only one direct 5-HT-acting compound has been launched as an anxiolytic agent (i.e. buspirone) (Goa and Ward, 1986; Apter and Allen, 1999). Nevertheless, interest in this research area has not diminished and novel 5-HT-modulating agents are still being developed (for review, see Griebel, 1997). Despite increasing interest in drugs combining 5-HT_{1A}, 5-HT_2 and/or 5-HT reuptake inhibitor properties, it is not yet clear whether the therapeutic potential of these agents will prove superior to that of selective 5-HT compounds. As such, research attention remains firmly focused on selective 5-HT_{1A} receptor ligands and, in this context, recent animal studies suggest that selective blockade of 5-HT_{1A} receptors may yield anxiolytic-like activity comparable to that of benzodiazepines (Cao and Rodgers, 1997a,b,c; Cao and Rodgers, 1998a,b; Griebel et al., 1999). For example, in the mouse elevated plus-maze test, several selective and non-selective 5-HT_{1A} receptor antagonists (WAY100135, WAY100635, *p*-MPPI, pindobind-5-HT_{1A}) have been shown to produce robust anxiolytic-like effects on both conventional (open arm activity) and

* Corresponding author. Tel.: +33-1-45-36-24-70; fax: +33-1-45-36-20-70.
E-mail address: guy.griebel@sanofi-synthelabo.com (G. Griebel).

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ethological (risk assessment) measures (Cao and Rodgers, 1997a,b,c; Cao and Rodgers, 1998a,b). Similarly, in a mouse defense test battery, where animals are directly confronted with a natural threat (i.e. a rat) as well as situations associated with this threat, selective 5-HT_{1A} receptor antagonists (WAY100635, SL88.0338) were found to modify defensive behaviors in much the same way as diazepam (Griebel et al., 1999). Furthermore, evidence for an anxiolytic-like action of 5-HT_{1A} receptor antagonists has also been reported in certain rat models of anxiety, such as the fear-potentiated startle (Joordens et al., 1998) and light/dark exploration (Sanchez, 1996) tests.

Despite these positive findings, however, there are a significant number of reports indicating that 5-HT_{1A} receptor antagonists are inactive in anxiety models. Thus, negative findings have been obtained in rat and pigeon conflict (Overshiner et al., 1995; Samanin et al., 1996; King et al., 1997; Millan et al., 1997; Kennett et al., 1998), rat ultrasonic vocalization (Bartoszyk et al., 1996; Brocco et al., 1996; Remy et al., 1996; Xu et al., 1997; Schreiber et al., 1998), rat conditioned emotional response (Overshiner et al., 1995; Stanhope and Dourish, 1996), mouse stress-induced hyperthermia (Olivier et al., 1998), rat social interaction (File et al., 1996) and rat elevated plus-maze (Bickerdike et al., 1995; File et al., 1996; Collinson and Dawson, 1997; Millan et al., 1997) tests. While some of these negative data may be due to the use of limited dose ranges, the general pattern of inconsistency has yet to be adequately explained. Based on the finding that the selective 5-HT_{1A} receptor antagonist, LY297996, produces anxiolytic-like activity in the murine elevated plus-maze in the mid-dark, but not the mid-light, phase, it has been suggested that circadian factors may be important in the detection of 5-HT_{1A} receptor antagonist anxiolysis (Cao and Rodgers, 1998a; Rodgers et al., 1998). Alternatively, as positive effects have largely been obtained in mouse models, and negative findings in rat models, the inconsistent profiles of 5-HT_{1A} receptor antagonists might be attributed to a species difference in the role of this receptor in anxiety-related processes.

The aim of the present experiments was to investigate the effects of several compounds varying in selectivity as 5-HT_{1A} receptor antagonists (WAY100635, SL88.0338, *p*-MPPI, MM-77 and pindobind-5-HT_{1A}) under identical test conditions in three well-validated rat models of anxiety. The tests chosen were two conflict procedures (punished lever-pressing and punished drinking) and one exploratory model (elevated plus-maze). Effects were directly compared to those of the prototypical anxiolytic diazepam, which was used as a positive control. We used different test procedures since there is now growing evidence that the measures of anxiety from different tests may reflect different states of anxiety (File, 1992; Belzung and Le Pape, 1994; Beuzen and Belzung, 1995; Rodgers, 1997). This was shown by the application of factor analysis of the various behavioral parameters obtained in different anxiety models. For example, File (1992) and Lister (1987) and Lister (1987) revealed that parameters recorded in several anxiety models (e.g. elevated plus-maze, social interaction, holeboard, Vogel conflict) produced distinct anxiety factors, thereby indicating that they reflect different emotional states.

The phenyl–piperazine derivative, WAY100635, and its close structural analogs, *p*-MPPI and the amino–methyl–piperidine SL88.0338, display high affinities for 5-HT_{1A} receptors (K_i=4.5, 1 and 2 nM, respectively), but only low to moderate affinities for α_1, D_2 and β receptors, and have demonstrated antagonistic-like activity at both somatodendritic 5-HT_{1A} autoreceptors and postsynaptic 5-HT_{1A} receptors (Kung et al. 1994, 1995; Zhuang et al., 1994; Fletcher et al., 1995; Assie and Koek, 1996; Thielen et al., 1996; Cohen et al., 1998). Unlike the pindolol derivative, pindobind-5-HT_{1A}, which displays antagonistic-like activity in both pre- and postsynaptic 5-HT_{1A} receptor models (Liau et al., 1991), MM-77 shows agonist-like activity at presynaptic somatodendritic 5-HT_{1A} receptors yet antagonistic-like activity in postsynaptic 5-HT_{1A} receptor models (Mokrosz et al., 1994). In addition, pindobind-5-HT_{1A} and MM-77 have only nine and two-fold selectivity for 5-HT_{1A} relative to α_1-adrenoceptors, respectively (Liau et al., 1991; Mokrosz et al., 1994).

2. Methods

2.1. Ethics

All procedures described here fully comply with French legislation on research involving animal subjects.

2.2. Subjects

Male Wistar rats (Charles River France, Saint-Aubin-les-Elbeuf) were used in the punished lever-pressing procedure. They weighed 180–200 g at the beginning of training and 400–500 g at the time of testing. Male Sprague–Dawley rats (Iffa Credo, L'Arbresle and Charles River France), weighing 180–300 g at time of testing, were used in the punished drinking (Vogel) and elevated plus-maze tests. Rats used in the Vogel procedure and the elevated plus-maze test were housed in groups of eight, whereas those used in the punished lever-pressing procedure were housed singly. The latter subjects were restricted to the food obtained during sessions together with a daily ration of 15–20g of standard laboratory chow given at the end of each weekday and over the weekend. All animals were maintained under

standard laboratory conditions (22–23°C) and kept on a 12:12-h light–dark cycle with light onset at 7 a.m.

2.3. Drugs

Diazepam, WAY100635 (N-{2-[4-(2-methoxyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride), SL88.0338 (4-((3,4-dihydro-5,8-dimethoxy-2(1*H*)-isoquinolinyl)methyl)-1-(3-ethoxybenzoyl)-piperidine) (synthesized by the CNS Chemistry Department, Synthélabo Recherche), pindobind-5-HT_{1A} (N^1-(bromoacetyl)-N^8-[3-(4-indolyloxy)-2-hydroxypropyl]-(Z)-1,8-diamino-*p*-menthane), *p*-MPPI (4-(2′-methoxyphenyl)-1-[2′-[N-(2″-pyridinyl)-*p*-iopobenzamido]-ethyl]piperazine) (RBI, Natick, USA) and MM-77 (1-(2-methoxyphenyl)-4-[(4-succinimido)butyl]-piperazine) (Tocris Cookson, Bristol, UK) were dissolved or prepared as suspensions in physiological saline containing one or two drops of Tween 80. Diazepam was administered intraperitoneally (i.p.), and WAY100635 was injected subcutaneously (s.c.) 30 min before experiments were carried out. The other drugs were given s.c. 15 min before the test. All doses are expressed as the bases and were chosen on the basis of previously published behavioral studies in mice (Bell and Hobson, 1993; Cao and Rodgers, 1997a,b,c; Griebel et al., 1999) and in rats (Stanhope and Dourish, 1996). All compounds were injected in a constant volume of 2 ml/kg.

2.4. Procedure

2.4.1. Punished lever-pressing

The procedure was a modification of that described previously (Sanger et al., 1985). Animals were tested in standard rat operant test chambers (MED Associates, Inc., GA) placed in sound-attenuated boxes with ventilation fans. Each chamber was fitted with a stainless-steel grid floor through which electric shocks could be delivered (shock generator and scrambler: MED Associates, Inc.). A total of 11 rats were trained initially to press a lever for food reward (45 mg precision food pellets, PJ Noyes, Inc., Lancaster). As training progressed, schedule parameters were gradually changed to a variable interval (VI) schedule (VI 30 s) of food reinforcement during daily 15 min sessions. After several sessions of VI 30 s responding, five 60 s periods of a visual stimulus were presented during a 25 min session. Each visual stimulus consisted of three stimulus lights situated above the food pellet dispenser and to the right of the response lever, which flashed at a rate of 1 s on, 1 s off. In this component, a footshock punishment schedule consisting of two independent VI schedules (VI 30 s for food, VI 10 s for shock) was in operation. Footshock was initially set at 0.1 mA. The first stimulus presentation started 5 min after the beginning of the session, and each following stimulus commenced 150 s after the end of the preceding stimulus. The magnitude of footshock was individually titrated for each rat (shock levels ranged from 0.3 to 0.65 mA) to obtain stable baselines of responding (i.e. an average lever pressing rate of 8 ± 2 presses in each 60 s punished responding period). To obtain stable levels of responding, an average of approximately 30 sessions after initiation of the punishment contingency was necessary. Once stable baselines of responding were obtained, drug studies were started.

Drug injections were given once or twice each week with at least two nondrug days intervening between two drug administrations. Vehicle was injected on all nondrug days. Drugs and doses were given in a mixed order, and treatment effects on punished and unpunished response rates assessed. The former corresponds to responses recorded during the presentation of the visual stimulus, whereas the latter were taken from the 60 s periods immediately preceding and immediately following each stimulus presentation. The mean values of punished and unpunished rates recorded during the nondrug session preceding the drug sessions were used as control scores. Drug effects were analyzed statistically by comparing performances after drug administration with the mean values taken from appropriate control sessions using a Friedman's ANOVA.

2.4.2. Punished drinking

The procedure was a modification of the technique described by Vogel et al. (1971). At the beginning of the experiment, rats, deprived of water for 48 h prior to testing, were placed in cages (27 × 22 × 21 cm) with a stainless steel grid floor. Each cage contained a drinking tube connected to an external 50 ml burette filled with tap water. Trials commenced only after the animal's tongue contacted the drinking tube for the first time. An electric shock (0.06 mA) was delivered through the drinking spout after every twenty licks, and the number of shocks received was recorded automatically during a 3-min period. Data were analyzed with one-way ANOVA. Subsequent comparisons between treatment groups and control were carried out using Dunnett's *t*-test.

2.4.3. Elevated plus-maze

The test apparatus was based on that described by Pellow et al. (1985). All parts of the apparatus were made of dark polyvinylplastic with a black rubber floor. The maze was elevated to a height of 50 cm with two open (50 × 10 cm) and two enclosed arms (50 × 10 × 50 cm), arranged so that the arms of the same type were opposite each other, connected by an open central area (10 × 10 cm). To prevent rats falling off, a rim of Plexiglas (1 cm high) surrounded the perimeter of the open arms. The illumination in the experimental room consisted of one red neon tube fixed on the ceiling, so that experiments were performed under dim light conditions. The light

intensity on the central platform was 10 lux. At the beginning of the experiment, rats were placed in the center of the maze, facing one of the enclosed arms, and observed for 4 min. The apparatus was equipped with infrared beams and sensors capable of measuring time spent in open arms, number of open-arm entries and number of closed-arm entries (defined as entry of all four limbs into an arm of the maze). In addition, rats were observed via video-link by an observer located in an adjacent room. This permitted the recording of the more ethologically-orientated measures: (a) attempt: attempt at entry into open arms followed by avoidance responses. This includes stretched attend posture (the rat stretches forward and retracts to original position); (b) head-dipping: protruding the head over the edge of an open arm and down towards the floor (this response can occur while the animal's body is in a closed arm, central square or on an open arm). The results were expressed as mean ratio of time spent in open arms to total time spent in both open and closed arms, mean ratio of entries into open arms to total entries into both open and closed arms, mean total number of both closed and open arm entries, mean total number of closed arm entries, mean total number of attempts and mean total number of head-dips. Data were analyzed by one-way ANOVA. Subsequent comparisons between treatment groups and control were carried out using Dunnett's t-test.

3. Results

3.1. Punished lever pressing

Fig. 1 shows that the rates of responding decreased by the punishment contingency were significantly increased by diazepam (χ^2=20.9, p<0.001), but not by the other compounds tested. The only compound to affect unpunished responding was WAY100635 which significantly decreased these response rates at 3 and 10 mg/kg (χ^2=24.3, p<0.001) (Table 1).

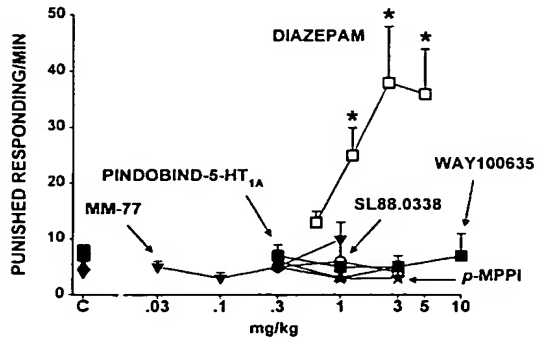

Fig. 1. Effects of diazepam and compounds varying in selectivity as 5-HT_{1A} receptor antagonists on rates of punished lever pressing in rats. Data represent mean±SEM. n=6–8. *p<0.05 (Dunnett's t-test).

3.2. Punished drinking

Fig. 2 shows that, except for pindobind-5-HT_{1A}, all compounds significantly modified the number of shocks received [diazepam: F(3,47)=10.4, p<0.001; WAY100635: F(3,39)=4.8, p<0.01; SL88.0338: F(3,79)=2.6, p<0.05; p-MPPI: F(4,49)=2.7, p<0.05; MM-77: F(4,53)=4.9, p<0.01]. Post-hoc analysis indicated that while diazepam (2.5 and 5 mg/kg), WAY100635 (0.3 and 1 mg/kg), SL88.0338 (3 and 10 mg/kg) and MM-77 (0.03–0.3 mg/kg) significantly increased punished responding at several doses, p-MPPI produced a significant effect at one dose only (1 mg/kg).

3.3. Elevated plus-maze

Fig. 3 shows that, with the exception of p-MPPI, all drugs significantly modified both the percentage of open arm time [diazepam: F(3,30)=7.6, p<0.001; WAY100635: F(4,65)=2.8, p<0.05; SL88.0338: F(4,33)=5.5, p<0.01; MM-77: F(4,65)=4.6, p<0.01;

Table 1
Effects of diazepam and compounds varying in selectivity as 5-HT_{1A} receptor antagonists on rates of unpunished responding in rats[a]

	Dose (mg/kg)	Unpunished responding/min
Diazepam	0	58±8
	0.625	74±9
	1.25	77±13
	2.5	76±10
	5	65±9
WAY100635	0	74±6
	0.3	65±8
	1	56±7
	3	49±8*
	10	23±7*
SL88.0338	0	70±8
	0.3	67±9
	1	64±9
	3	60±10
p-MPPI	0	67±5
	0.3	61±8
	1	67±5
	3	64±9
MM-77	0	51±5
	0.03	57±5
	0.1	48±6
	0.3	55±8
	1	41±7
Pindobind-5-HT_{1A}	0	62±10
	0.3	58±10
	1	54±15
	3	48±5

[a] Data represent mean±SEM. n=6–8. *p<0.05 (Friedman).

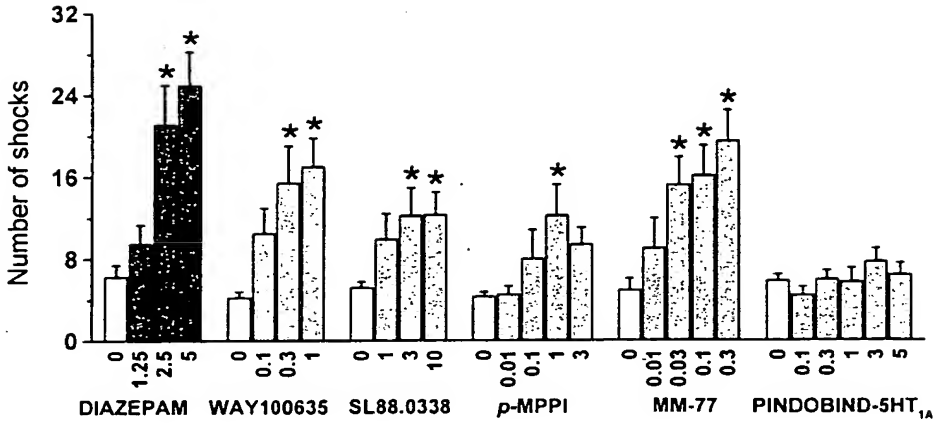

Fig. 2. Effects of diazepam and compounds varying in selectivity as 5-HT_{1A} receptor antagonists in the punished drinking conflict test in rats. Data represent mean±SEM. n=10–20. *p<0.05 (Dunnett's t-test).

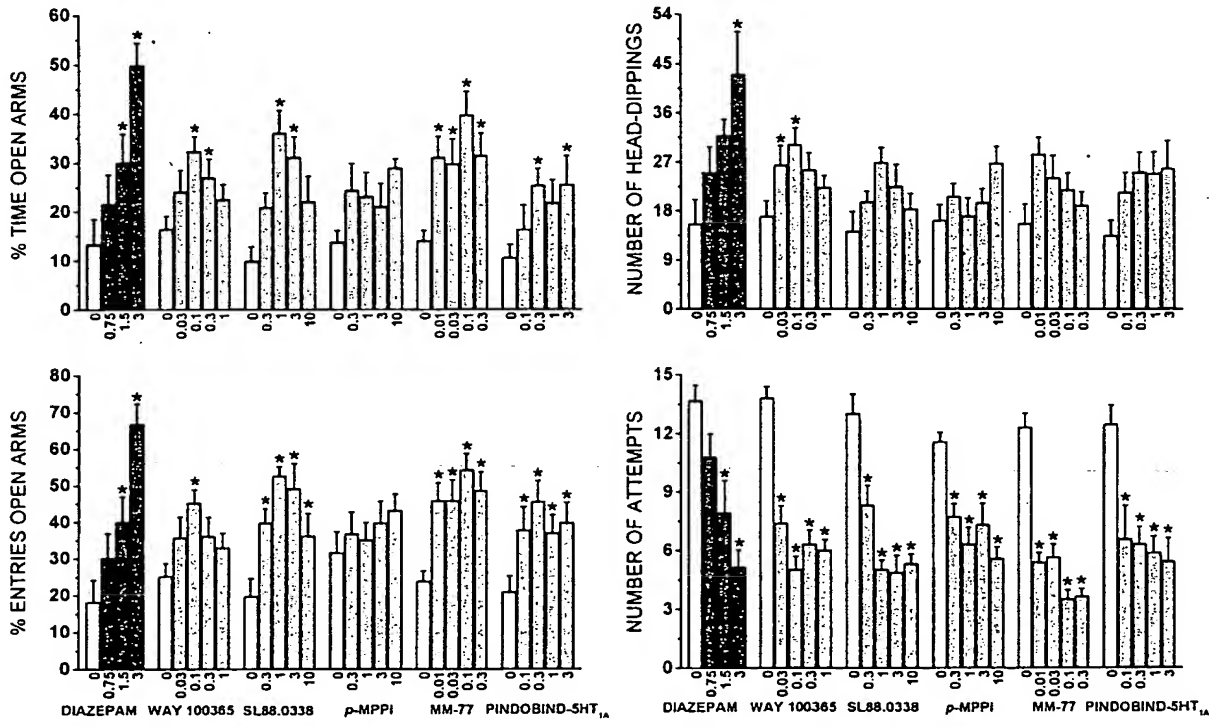

Fig. 3. Effects of diazepam and compounds varying in selectivity as 5-HT_{1A} receptor antagonists on four anxiety-related measures in the elevated plus-maze test in rats. Data represent mean±SEM. n=6–14. *p<0.05 (Dunnett's t-test).

pindobind-5-HT_{1A}: $F(4,32)=2.7$, $p<0.05$] and the percentage of open arm entries [diazepam: $F(3,30)=9.8$, $p<0.001$; WAY100635: $F(4,65)=2.5$, $p<0.05$; SL88.0338: $F(4,33)=5.8$, $p<0.01$; MM-77: $F(4,65)=5.8$, $p<0.001$; pindobind-5-HT_{1A}: $F(4,32)=3.6$, $p<0.001$]. Post-hoc analysis indicated that each of these drugs significantly increased open arm activity at several doses. With respect to the ethologically-derivated measures, all compounds modified the number of attempts at entry into open arms followed by avoidance responses [diazepam: $F(3,30)=8.9$, $p<0.001$; WAY100635: $F(4,65)=24.5$, $p<0.001$; SL88.0338: $F(4,33)=17.2$, $p<0.001$; p-MPPI: $F(4,34)=8.8$, $p<0.001$; MM-77: $F(4,65)=39.3$, $p<0.001$; pindobind-5-HT_{1A}: $F(4,32)=7.7$, $p<0.001$]. Post-hoc analysis indicated that all compounds significantly reduced attempts over a

wide dose-range. In addition, diazepam [$F(3,30)=3.3$, $p<0.05$] and WAY100635 [$F(4,65)=2.8$, $p<0.05$] modified directed exploration (head-dippings). Post-hoc analysis revealed that this response was significantly increased by diazepam at 3 mg/kg and by WAY100635 at 0.03 and 0.1 mg/kg. Finally, Table 2 shows that only diazepam [$F(3,30)=7.7$, $p<0.001$] and MM-77 [$F(4,65)=5$, $p<0.01$] significantly decreased the number of closed arm entries, whereas none of the drugs significantly modified the total number of arm entries.

4. Discussion

The present study compared the behavioral profiles of compounds varying in selectivity as 5-HT_{1A} receptor antagonists with those of diazepam in three classical rat models of anxiety. As expected, diazepam was active in all three models, increasing rates of punished lever-pressing, punished drinking and open arm activity. However, the effects of the 5-HT_{1A} receptor antagonists varied according to the test employed.

Table 2
Effects of diazepam and compounds varying in selectivity as 5-HT_{1A} receptor antagonists on two measures of general activity in the elevated plus-maze test in rats[a]

	Dose (mg/kg)	Closed arm entries	Total arm entries
Diazepam	0	10.4±0.9	12.8±0.8
	0.75	10.1±0.7	14.8±0.6
	1.5	8.3±1	14.4±1.6
	3	4.6±1.1*	13.1±1.4
WAY100635	0	11.6±1.3	15.4±1.3
	0.03	8.8±0.7	14.1±0.5
	0.1	8.5±0.6	15.5±0.6
	0.3	9.8±0.8	15.6±0.9
	1	10.6±1.2	15.6±1.1
SL88.0338	0	9.5±0.9	12.3±1.8
	0.3	7±0.7	11.7±1
	1	5.7±0.4	12.1±1
	3	6.4±0.9	12.6±1
	10	6.9±1.1	10.9±1.4
p-MPPI	0	9.6±1.4	14.1±1.5
	0.3	8.1±0.6	13.1±1
	1	8.3±0.4	13±0.9
	3	8.3±1.3	13.6±1.3
	10	7.1±0.6	12.6±0.6
MM-77	0	10±0.9	13±1
	0.01	7.8±0.8	14.7±1
	0.03	7.5±0.9	14.3±1.2
	0.1	6.1±0.6*	13.1±1
	0.3	5.6±0.6*	11.4±0.9
Pindobind-5-HT_{1A}	0	10.2±0.9	13.2±1.2
	0.1	7.4±1.1	11.7±0.9
	0.3	8.1±0.4	15.6±1.3
	1	8.9±0.8	14.3±1.4
	3	9.2±1.7	15.2±2

[a] Data represent mean±SEM. n=6–14. *$p<0.05$ (Dunnett's t-test).

In the punished lever-pressing conflict test, none of the 5-HT_{1A} receptor antagonists modified rates of punished responding, whereas in the punished drinking test, WAY100635, SL88.0338, MM-77 and, to a lesser extent, *p*-MPPI (but not pindobind-5-HT_{1A}) produced anticonflict activity. In addition, the observation that WAY100635 decreased unpunished responding at 3 and 10 mg/kg is in agreement with a previous finding in mice that this compound induces immobility at a dose of 9 mg/kg (Cao and Rodgers, 1997c). The general absence of significant modifications in rates of unpunished responding in the lever-pressing procedure (at doses active in the punished drinking test) indicates that the anxiolytic-like effects on punished drinking were observed at doses which did not impair motor activity. However, it is important to note that the increase in punished responding with the 5-HT_{1A} compounds was somewhat smaller than that produced by diazepam, indicating weaker anxiolytic-like activity. It is unlikely that the positive effects of 5-HT_{1A} receptor antagonists in the punished drinking test are due to decreased sensitivity to electric shocks since these drugs have been reported to be inactive in reflexive tests of analgesia (i.e. the tail-flick an hot-plate), irrespective of stimulus quality or intensity (Millan, 1994). Although pindobind-5-HT_{1A} was inactive in both conflict tests, it is possible that doses higher than 5 mg/kg may have been more effective. However, in a previous study the drug was shown to elicit anxiolytic-like effects in mice from 0.1 to 0.5 mg/kg (Cao and Rodgers, 1997b) indicating that, under certain test conditions, this compound can modify anxiety-related behaviors at doses lower than 5 mg/kg.

The failure of 5-HT_{1A} receptor antagonists to modify punished lever-pressing, while entirely consistent with previous findings in rat and pigeon conflict tests (Overshiner et al., 1995; Samanin et al., 1996; King et al., 1997; Millan et al., 1997), is difficult to reconcile with the positive effects obtained in the punished drinking (Vogel) test. Moreover, our data contrast with those obtained by Kennett et al. (1998) in the Vogel conflict test. In this study WAY100635 was found inactive at 0.1 and 0.3 mg/kg, whereas we found effects with somewhat higher doses (0.3–1.0 mg/kg). Thus dose range could be the problem here as could differences in control levels of punished drinking (high in Kennett's study vs us: around 9 vs 4 shocks accepted). In addition, Kennett et al. used a very different test procedure. For example, their deprivation schedule was different. Further, in their study, a pre-test was performed one day prior to testing, suggesting that animals were less stressed than those used here, that didn't see the test apparatus before. Overall, it seems likely that these models may be tapping different facets or levels of anxiety. Thus, it is not unreasonable to assume that the level of stress in the punished drinking test is higher than that in the punished lever pressing procedure. In the former, the experimental

situation was novel to the rats and they had never experienced electric shock prior to testing. In the latter, however, animals had been handled daily and extensively trained (several months) in the same cage and were fully experienced with electric shock before drug testing. This distinction suggests that the increase in 5-HT release, generally produced by exposure to aversive stimuli (e.g. Blanchard et al., 1991; Bickerdike et al., 1993; File et al., 1993; Kawahara et al., 1993; Shekhar et al., 1994; Yoshioka et al., 1995), may well be lower in the lever-pressing procedure than in the punished drinking test. Thus, assuming that endogenous 5-HT tone contributes significantly to the emotional responses displayed by rats in the latter test, 5-HT_{1A} antagonists would be predicted to attenuate these reactions. Although there is as yet no direct evidence that these conflict tests differentially modify 5-HT release, it is notable that the α_2-adrenoceptor ligand, yohimbine, which exhibits marked activity at 5-HT_{1A} receptors (Winter and Rabin, 1992), has been found to exert anticonflict activity in the rat punished drinking test by decreasing 5-HT neurotransmission (Soderpalm et al., 1995a,b). Alternatively, the discrepancy between both conflict tests may be related to the different housing conditions used with these tests. While rats used in the lever pressing test were housed singly, those employed in the punished drinking test were housed in groups of eight. Based on the finding that housing conditions affect the 5-HT system (Crespi et al., 1992), it is possible that a different 5-HT regulation between these rats may lead to changes in the sensitivity to 5-HT_{1A} receptor antagonists.

In the elevated plus-maze, and fully consistent with previous findings in the mouse version of this test (Cao and Rodgers, 1997a,b,c; Cao and Rodgers, 1998a,b), all drugs showed anxiolytic-like activity on traditional behavioral indices i.e. increases in percentage open arm entries and time. However, despite trends in the appropriate direction, the effects of *p*-MPPI were not statistically significant. The reason for this is unclear, as previous work has reported robust anxiolytic-like effects with this compound in the mouse elevated plus-maze (Cao and Rodgers, 1997a). However, it is pertinent to note that, in the mouse defense test battery, *p*-MPPI also elicited weaker anxiolytic-like effects than either WAY100635 or SL88.0338 (Griebel et al., 1999). Importantly, effects of the 5-HT_{1A} ligands on spatiotemporal measures occurred at doses that did not decrease closed or total arm entries (reliable measures of locomotor activity), thereby suggesting that the anxiolytic-like activity was not contaminated by motor impairment. However, as for the punished drinking test, the magnitude of the effects observed with the 5-HT_{1A} compounds on conventional plus-maze measures was generally smaller than that of diazepam. A similar potency differential has recently been reported in a direct comparison of the effects produced by chlordiazepoxide and WAY100635 in the mouse elevated plus-maze test (Cao and Rodgers, 1998b). The behavioral profile of WAY100635 in this study contrasts with several previous findings in rats showing that the drug failed to modify open arm activity (Bickerdike et al., 1995; File et al., 1996; Collinson and Dawson, 1997; Millan et al., 1997). This variability cannot be attributed to dose range as doses currently used overlap with those employed in previous investigations. Similarly, the discrepancy cannot be explained by strain differences (Sprague–Dawley rats were used in two studies) or by differences in route of injection/injection-test intervals (similar in all studies). However, it is important to note that, in previous work, baseline levels of time spent on the open arms were above 20% whereas, in the present study, values ranged between 10 and 16% (i.e. slightly higher baseline anxiety). Assuming that basal release of 5-HT increases as a function of the degree of stress experienced, it could be predicted that current test conditions would be more likely (than those operating in previous research) to reveal behavioral activity for 5-HT_{1A} receptor antagonists (e.g. see Hogg, 1996).

Regarding the ethological plus-maze measures, all compounds markedly decreased risk assessment (i.e. attempts) over a wide dose-range, but only diazepam clearly increased directed exploration (i.e. head-dipping). Benzodiazepine-induced stimulation of head-dipping in exploratory models of anxiety has been widely reported in the literature (e.g. Cole and Rodgers, 1993; Shepherd et al., 1994; Griebel et al., 1996; Cao and Rodgers, 1998b). Although WAY100635 also significantly increased head-dipping, the magnitude of this effect was more comparable to the small increase seen with the other 5-HT_{1A} receptor ligands than to the robust effect of diazepam. The results for attempts further confirm that this risk assessment response is particularly sensitive to the action of 5-HT_{1A} receptor ligands (Rodgers et al. 1994, 1995; Griebel et al., 1997; Setem et al., 1999). Interestingly, comparisons with 5-HT_{1A} receptor agonists previously assessed in the elevated plus-maze in our laboratory indicate some potentially important differences. Thus, full agonists (8-OH-DPAT and flesinoxan) and partial agonists (buspirone and ipsapirone) have all been found to affect the behavior of rats in this test (Griebel et al. 1997, 1998). However, while producing clear reduction in attempts, they failed to modify the conventional open arm avoidance measures. Taken together with findings in the murine plus-maze (Cao and Rodgers, 1997a,b,c) and defense test battery (Griebel et al., 1999), present results suggest that the anxiety-reducing potential of 5-HT_{1A} receptor antagonists may be superior to those of either full or partial agonists for this receptor.

Overall, the results of the present series of experiments demonstrate that 5-HT_{1A} receptor antagonists can produce anxiolytic-like effects in rats. However, these

effects appear to be test-specific and, unlike results obtained in mice, generally weaker than those produced by the prototypical anxiolytic, diazepam. This profile, along with dose–response and rate-dependency considerations, may partially explain inconsistent findings with these agents in previous studies using rat models of anxiety. Furthermore, the apparently greater consistency of effect observed in mouse versus rat models may be related to a species difference in the molecular pharmacology of the 5-HT_{1A} receptor subtype. In this context, and while there is little evidence of a major species difference in the central distribution of 5-HT_{1A} receptors (Pazos and Palacios, 1985; Waeber et al., 1989), in vivo studies would suggest an important species difference in 5-HT_{1A} receptor function. For example, in mice, 8-OH-DPAT-induced hypothermia is mediated by presynaptic 5-HT_{1A} autoreceptors, whereas in rats, it might be mediated by postsynaptic 5-HT_{1A} receptors (Bill et al., 1991). It is therefore conceivable that distinct 5-HT_{1A}-mediated mechanisms may underlie the behavioral responses to 5-HT_{1A} receptor antagonists in rat and mouse models of anxiety. However, given the general similarity of effect observed in rat and mouse versions of the same test (i.e. plus-maze), an alternate interpretation is that the extent to which 5-HT_{1A} receptor mechanisms are involved in the regulation of anxiety may depend critically upon the precise nature of the response studied.

Several recent studies using gene-targeting technology to generate 5-HT_{1A} receptor knockout mice have shown that these animals display more anxious-like behaviors (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). These results differ from those obtained in the present study where 5-HT_{1A} receptor blockade leads to an opposite action. However, the "chronic" blockade of 5-HT_{1A} receptors in mutant mice can hardly be compared to an acute blockade of these receptors by an antagonist. The lack of 5-HT_{1A} receptors in mutant mice may have produced developmental compensations which compromise a direct comparison between both types of studies.

The precise mechanisms underlying the positive effects of 5-HT_{1A} receptor antagonists in anxiety models remain to be determined. The compounds used in this study have all demonstrated antagonistic-like activity on postsynaptic 5-HT_{1A} receptors. It it is therefore possible that this mechanism may underlie the anxiolytic-like effects of these compounds. In addition, based on the findings that exposure to aversive stimuli like those used in the above studies increases 5-HT release, we would expect a 5-HT_{1A} receptor antagonist to attenuate this effect and thus display anxiolytic activity. However, further studies are clearly warranted to determine why these compounds failed to be active in several models of anxiety.

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